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LARGE MOLECULES THROUGH ATOMIC SPECTACLES

HUGH S. TAYLOR

David B. Jones Professor of Chemistry, Princeton University
(Franklin Medal Lecture, April 24, 1941)

ABSTRACT

The highest resolutions now attainable with the electron microscope permit us to examine objects which are about 40 Ångström units in linear dimension. Interatomic distances are, however, of the order of 1 to 3 Ångström units so that to examine the geometry of the constituent units of molecules it is still necessary to construct and study models made to scale in accordance with the best data of physico-chemical science. The Fisher-Hirschfelder atomic molecules have proved especially useful in this problem of viewing molecules through atomic spectacles. The method of attaching atoms one to another provided by the makers of these models is not very satisfactory. A new method of attachment has been developed which uses the well-known snap-fastener of the rigid head-resilient socket type to produce an attachment which is "positive," or locked against accidental rupture, while permitting full and free rotation between singly bonded atoms. Molecular models so produced have manipulative stability and a flexibility of motion more closely corresponding to the behavior of the molecular structures modelled. In use, these reveal striking results on the folding of polypeptide chains, such as silk and hair and of other fibrous structures. Folding in three dimensions indicates the possibility of many types of orientation of the peptide links with room for large and small side-chains. Models of complex molecules such as the vitamins, sterols, carcinogenic agents, plastics and rubber polymers reveal characteristics of such molecules which are not immediately evident from the older structural formulæ of the chemist.

In the laboratory notebooks of John Dalton in the library of the Manchester Literary and Philosophical Society the earliest efforts to visualize molecules through atomic spectacles were carefully preserved. An entry dated September 6, 1803, gives the following characters of elements

○ Hydrogen	● Carbone, pure Charcoal
⊙ Oxygen	⊕ Sulphur
◑ Azote	

and, a few pages further on, the following examples of Dalton's method of expressing the composition of chemical compounds

◑⊙◑ Nitrous Oxide	○⊙ Water
◑⊙ Nitrous Gas	◑○ Ammonia
⊙◑⊙ Nitric Acid	⊙● Gaseous Oxide of Carbon
⊙◑⊙ Nitrous Acid	⊙●⊙ Carbonic Acid
◑⊙	⊙⊕⊙ Sulphuric Acid
⊕⊙ Sulphurous Acid	

On December 23 last, during a German air-raid on Manchester, the home of one of our sister societies was subjected to wholesale destruction by bomb and incendiary. These historical documents of the beginnings of the modern era in chemistry, the atomic era, may well have fallen victims to "enemy action".

Dalton's symbol for water ○⊙ is significant. In modern dress this would be HO whereas we now write it H_2O. Avogadro had enunciated as early as 1811 the principles that determined which of these alternatives was the correct one but it was not until his fellow-countryman Cannizaro demonstrated in 1858 the applicability of Avogadro's ideas that general agreement among chemists was reached and the formulæ, which we still use today, were universally accepted. The analytical chemists of the first half of the 19th century provided the quantitative analyses from which the atomic composition of a wide variety of inorganic compounds could be deduced with the aid of the Dalton concept of atoms and atomic weights. With the rise of organic chemistry under Dumas, Liebig, Wohler, Williamson, Frankland and others, organic compounds were first classified in terms of their corresponding types. Thus, hydrocarbons were of the hydrogen type, organic chlorides of the hydrochloric acid type, ethers of the water type and amines of the ammonia type. Gradually the idea of valence in organic chemistry was recognized and carbon shown to be tetravalent as most simply illustrated in methane CH_4. Structural formulæ were developed in which a two-dimensional picture of the component atoms expressed the four valencies of the carbon as typified in such formulæ as propane $CH_3 \cdot CH_2 \cdot CH_3$, methyl ether $CH_3 \cdot O \cdot CH_3$, propyl chloride $CH_3CH_2CH_2Cl$, propyl amine $CH_3 \cdot CH_2 \cdot CH_2 \cdot NH_2$ and unsaturation was recognized in compounds with double and triple bonds such as ethylene $H_2C=CH_2$ and acetylene $HC \equiv CH$. Later it became clear that molecules having the same atomic content might have different arrangements of atoms, that is to say "isomers" such as $CH_3 \cdot CH_2 \cdot CH_2Cl$ and $CH_3 \cdot CHCl \cdot CH_3$

could exist. Pasteur then recognized that, in the crystals of tartaric acid, there was the phenomenon of left-handed- and right-handedness, that this implied an isomerism in space. van't Hoff and LeBel embodied this concept of stereo-isomerism in the concept of a spatial arrangement of the four valencies of carbon; for example, in methane, the carbon atom was located at the center of a tetrahedron, the hydrogen atoms at the corners of the tetrahedron. Upon that conception of atoms in space the organic chemist worked out by pure processes of reasoning an accurate body of knowledge as to the disposition of atoms in many molecules.

Deductive processes such as these were necessary since the available physical tools were not as yet adequate to the location of atoms in a molecular structure. With a microscope having a good immersion-system it is only possible to resolve details of a size somewhat less than two ten-thousands of a millimeter using ordinary daylight as the light source. An object of such a linear dimension would have as many as a thousand or more atoms side by side in a row. With the invention of the ultramicroscope and using ultraviolet light as the source of illumination, this resolution could be doubled, but, even so, the magnification was still more than 500 times too feeble. The resolution attainable with the electron microscope, the newest tool in the microscope field, can be pushed to 40 Ångström units (10^{-8} cm.) or to approximately 20 atom diameters. To be able to view the individual atoms it would therefore be necessary to improve the resolution still another twenty-fold.

X-Ray Analysis of Crystal Structure

It was in 1912 that the first development occurred which permitted the physicist and the chemist definitely to locate the position of atoms in crystalline materials. Laue in Germany and the Braggs in England independently employed the X-rays to discover the orderly arrangement of atoms and molecules, various estimates having placed these atoms in crystals at distances of the order of 10^{-8} cm. This was the right distance for the reflection and diffraction of the short wave-length of the X-rays, the atoms serving the same purpose with these rays that gratings, ruled by hand, had served Rowland and others in the resolution of ordinary light waves. These developments served a dual purpose; they showed that X-rays were in reality light of short wave-length but they also permitted scientists to put on their atomic spectacles, see the actual architecture of crystalline materials and learn that the external form of those crystals, their properties of cleavage, indicated an internal structure consisting of regular arrangements in space of ultimate units, the constituent atoms.

Sodium chloride was revealed as a cubical structure with atoms or rather ions of sodium and chlorine occupying alternately positions at the corners of a cubical structure or lattice (Fig. 1).

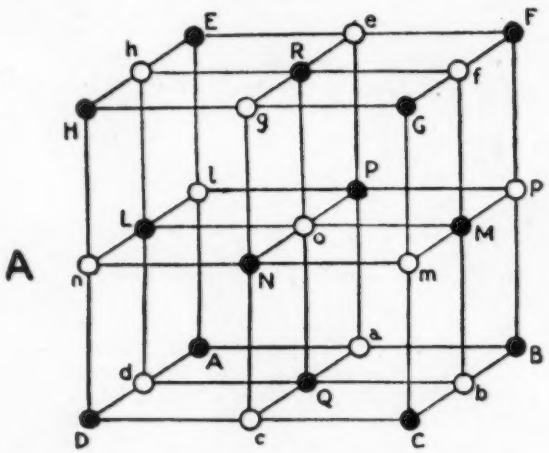

Fig. 1. Cubic lattice of sodium chloride. From W. H. and W. L. Bragg, The Crystalline State, Bell, London, 1933.

The structure of diamond was found to confirm the organic chemist's concept of the four-valency of carbon, directed tetrahedrally in space, each carbon atom occupying a position central to four neighbors distributed around it at the corners of a regular tetrahedron and at distances of 1.54 Å units (Fig. 2). That the architecture of the crystal reveals its properties is well illustrated by the case of graphite in the same diagram. Here the atoms were found to be arranged in flat plates of hexagonal rings each atom in the ring at the same distance (1.42 Å) from its neighbors. These plates, however, in contrast to the case of diamond, are relatively far apart, 3.25 Å or over twice the spacing of adjacent planes in diamond. It is between these planes that the attractive forces are smallest and it is along these planes that the well-known cleavage of graphite occurs. Flaky, aromatic crystals such as benzene, naphthalene and anthracene possess these same hex-

agonal ring planes with the same dimensions. Paraffins, aliphatic hydrocarbon chains, possess the carbon-carbon distance 1.54 Å found in diamond.

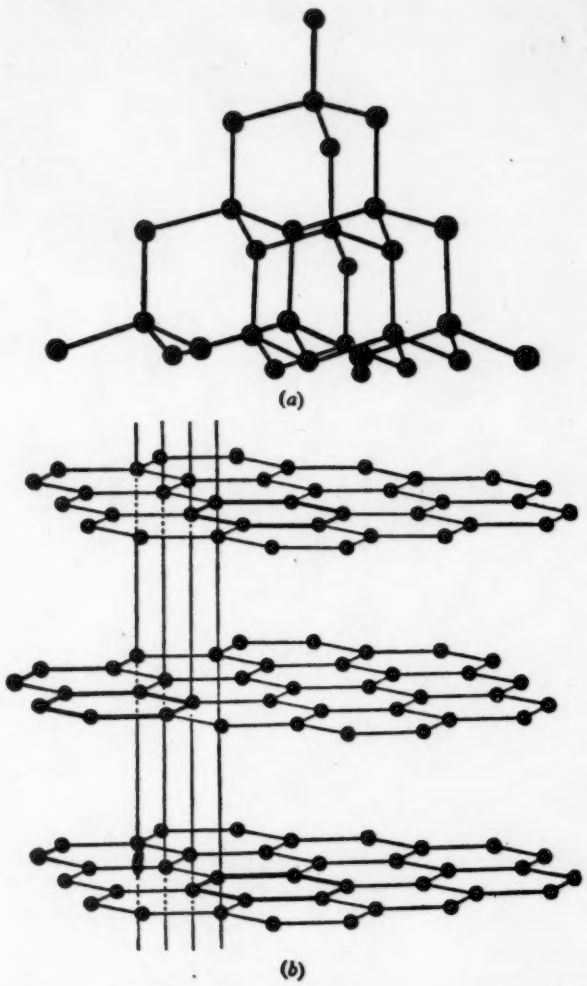

FIG. 2. Diamond and graphite structures. From W. H. and W. L. Bragg, The Crystalline State, Bell, London, 1933.

This conclusion from X-ray analysis found a very striking confirmation in the beautifully simple experiments of Langmuir on the areas covered by fatty acids spread as a monolayer, a layer of single molecules, on the surface of water. Fatty acids are long chains of hydrocarbons with a polar ending, COOH. The polar end is attracted to the water surface and the hydrocarbon chain is oriented out of the water and at a slight tilt from the vertical. By spreading known volumes of these fatty acids on water surfaces, measuring the area covered, Langmuir could deduce the cross-sectional area and, hence, the length of the hydrocarbon chain. These experiments proved independently that each carbon atom in the chain was at the diamond-distance, 1.54 Å from its neighbors, and that the whole chain was the zig-zag arrangement that the concept of the tetrahedral carbon atom would demand.

ELECTRON DIFFRACTION OF MOLECULES

The developments of quantum theory taught us the dualistic nature of light, its behavior in certain circumstances as waves, its atomistic nature as units or quanta of light in other circumstances. The application of this dualistic concept to matter in the early 1920's led to the consideration of matter not only as particulate or atomistic but also as waves and this in its turn led to the striking experiments, by Davisson and Germer and independently by G. P. Thomson, using electrons where X-rays had previously been used and, from the pattern of the electron reflections and diffraction, confirmation was obtained of the unitary structure of matter. The principal advantage deriving from electron diffraction experiments is that it has permitted the study not only of crystals but of liquids and, what is of still greater importance, the study of the atomic structure of gases and vapors. By means of such measurements we now have a large body of experimental data on the position of atoms in molecules, their distances one from another and the angles between the different constituent atoms in the molecule. We thus have from X-rays and electron diffraction studies, supplemented by a large body of data from the study of band spectra of molecules, a most detailed body of knowledge concerning the sizes of the individual atoms in molecules and ions in crystals as well as the variations in the atomic distances with changes in the manner in which these atoms are linked together.

The diagrammatic representation of these crystal and molecular structures usually presents a lattice framework, the centers of gravity of the atoms represented by circles or points and these joined by lines of length corresponding to the known atom distances. Actually it is now known that the atoms or ions enter into the structure as if they were spheres of definite size. To each atom or ion may be assigned an atomic

or ionic radius, the distance between two centers being equal to the sum of the two radii. On this view the crystal or molecule becomes a close, ordered arrangement of spherical units the sizes of the units determining the geometry of the whole crystal. The forces that hold the units together vary. In inorganic crystals, such as sodium chloride, the bond is frequently ionic in character, the electrical attractions between oppositely charged particles being the most important binding forces. In other structures, the bond is a valency bond, the so-called co-valent bond, as in diamond, electrically neutral atoms being held by the same valency forces that hold atoms together in molecules. Other crystals, for example those of dry ice, are held together by neither ionic nor valency bonds, but by the weaker forces, the van der Waals forces, that hold molecules together. It is the molecules rather than atoms which are built into the geometrical structure. Such crystals have relatively low melting points and boiling points, as is well illustrated by dry ice. Most metals and alloys form structures of close-packed spheres of positively charged metal ions in which the free electrons, that give the metallic characteristics of heat and electrical conduction, are freely moving.

Structures Containing Silicon and Oxygen

Let us put on our atomic spectacles, now, and look at some of the structures that arise from the linking of atoms together in architectural patterns dominated by well-defined and ascertained rules of structure building. First of all we shall examine some of the possibilities that arise when the two very common elements in nature, silicon and oxygen, interact. We choose this as our starting point for several reasons: first, because only two types of atoms are involved; secondly, because they interact according to a very simple rule and yet are able to produce patterns of structure with which we are all familiar, which confer valuable properties on the materials so constructed; and lastly, these structures may be used as type-illustrations of systems much more complex in composition and of great importance in organic and biological systems.

Silicon is always found between four oxygen atoms.

(*a*) As an independent group, SiO_4, tetrahedral in structure, we find it associated as a negative ion, having four negative valencies, with metal ions in various silicates, *e.g.*, Forsterite, $Mg_2(SiO_4)$, Zircon, $ZrSiO_4$, Garnet, $Ca_3Al_2(SiO_4)_3$, Topaz, $(AlF)_2SiO_4$.

(*b*) It may form closed systems, two tetrahedra linked together by a common oxygen atom and thus in units of Si_2O_7, or three in a ring with three common oxygen atoms in a unit Si_3O_9, or six in a ring with six common oxygens in a unit Si_6O_{18}. This last unit is to be found in the beryl $Be_3Al_2Si_6O_{18}$.

(*c*) It may form endless chains and bands. The simplest type of chain is a string of tetra-

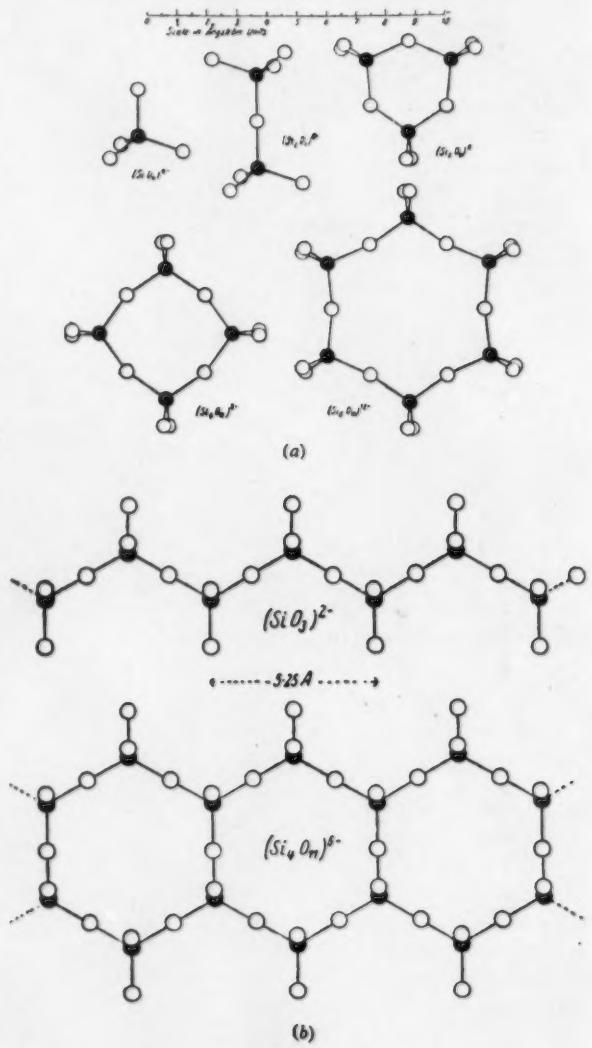

Fig. 3. Types of silicon-oxygen grouping in the silicates (*a*) Closed groups (*b*) Chains and bands. From W. H. and W. L. Bragg, The Crystalline State, Bell, London, 1933.

hedra linked corner to corner with an oxygen atom common to each linked tetrahedron. The composition of such a chain would be $(SiO_3)_n$. Two chains side by side and cross-linked would form a band. Minerals with these characteristic arrangements are fibrous in character. Asbestos is such a mineral. The long chains are bound together laterally by metallic ions.

These three types of linkage are illustrated in Fig. 3.

(*d*) Sheets of linked tetrahedra, each tetrahedron sharing three corners, with a sheet composition $(Si_2O_5)_n$, are present in laminar minerals such as talc and mica. They cleave readily, as does graphite, parallel to the planes of sheets which are held together by metallic ions.

(*e*) Three dimensional networks arise when each tetrahedron shares each of its corners. Pure silica is of this form with a composition $(SiO_2)_n$. Silicon may be partially replaced by aluminum, the resulting unit $(Si, Al)O_2$ having a resultant negative charge, requiring the incorporation into the structure of metallic ions. Felspars are of this type and in the zeolites we have a silica-alumina structure so open that the basic ions may be withdrawn from the structure by replacement, a property made use of in permutit water-softening devices. The three-dimensional network may be cage-like, the well-known ultramarine pigment being of this type.

The linkages discussed in (*d*) and (*e*) are illustrated in Fig. 4. W. L. Bragg in England and L. Pauling in this country are largely responsible for our detailed knowledge of these structures which may assume great complexity. In the majority of them the crystals are ionic in character.

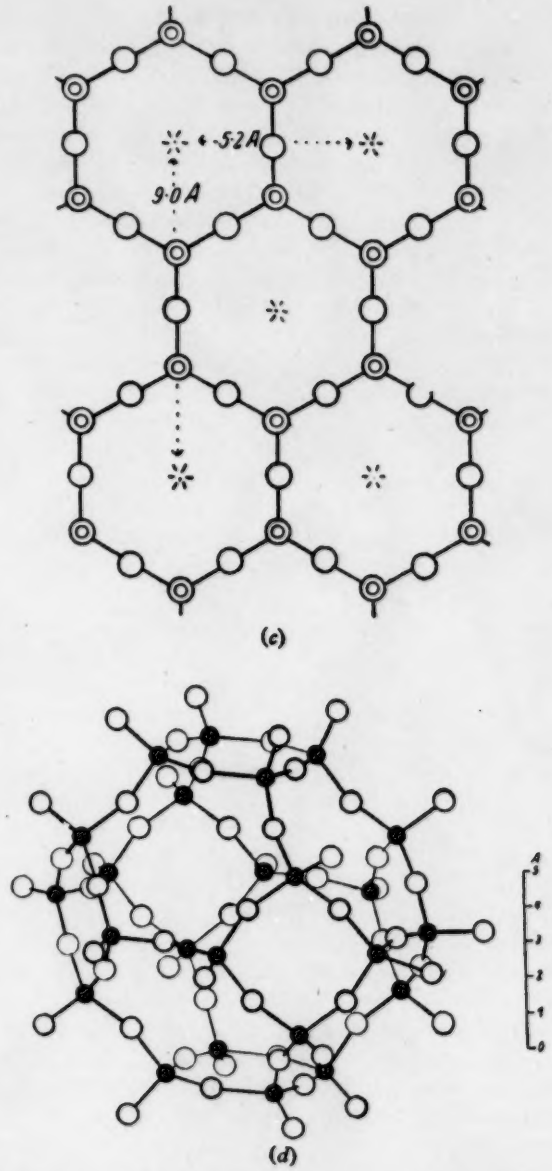

FIG. 4. Types of silicon-oxygen grouping in the silicates (*c*) Sheet (mica) (*d*) Three-dimensional net (ultramarine). From W. H. and W. L. Bragg, The Crystalline State, Bell, London, 1933.

COMPLEX BIOCHEMICAL STRUCTURES

To attain structures of parallel complexity in which the binding is of a co-valent character it is necessary to turn to the realm of organic and biological chemistry. Here the capacity of carbon to link up with itself and other atoms in bonds of the usual valence type gives rise to structures, *in vitro* and *in vivo*, of a complexity outranking that of the inorganic world, the solution of which problems is even yet a matter for conjecture and the most fascinating research. In the simpler organic systems, by processes of breakdown and of synthesis, coupled with a most penetrating deductive effort, the structures have been quantitatively decided. This is true even for the vitamins, the sterols and the hormones. In the more complex systems, both synthetic and natural, for example in the high-molecular weight synthetic resins and plastics, now so familiar, and especially in the more highly organized natural products such as rubber, cellulose fibers such as cotton, ramie and wood, starch,

fibrous proteins such as silk, wool and the muscle proteins, globular proteins such as egg albumen, hæmoglobin and insulin, right up to such complex structures as the tobacco mosaic virus, with molecular weights ranging from the tens of thousands to many millions, we are only at the initial stages of solution of the problem.

Molecular Structures from Model Atoms

The ability to penetrate into the intricacies of such structures has been tremendously facilitated by the construction of atomic models based upon the findings of the X-ray, spectroscopic and electron diffraction data furnished by modern science as to the sizes of atoms and the included angles when assembled into molecules. From the determinations made upon hundreds of compounds, tables of data have been compiled from which atom-models of the correct dimensions with correct valence angles for particular valence states have been constructed. Recently such models have been introduced commercially by the Fisher Scientific Company with the coöperation of Professor J. O. Hirschfelder. They conform to the best data on atom-diameters, faces and angles between valence bonds and yield the correct relationships as to numbers of bonds per face and the planes in which they lie. With such atom models, even in the case of simple organic molecules, a much more instructive portrayal of molecular properties can be secured than is possible with the two-dimensional formulæ of earlier days.

With the various atomic units now available, including four types of carbon atom, two of oxygen, four of nitrogen, three of sulfur and one each of chlorine, bromine, iodine and hydrogen—the multiple forms for a given atom representing different types of bonding—it is possible not only to construct the simpler molecules with a few atoms but complex structures containing hundreds of constituent atoms. The linear scale of the atoms is so chosen that 1 cm. represents one Ångström unit (10^{-8} cm.) or a scale of 100 millions to one. One result of this choice of scale is that, with the aid of a centimeter rule the linear dimensions of the completed model can be rapidly measured with a reasonable degree of accuracy.

The atomic units, as purchased, are assembled and held together by the use of double taper pegs. While these are adequate for the assembly of the simpler structures it has been quite generally found that larger molecular units do not possess an adequate degree of stability in handling subsequent to assembly. The weakness lies in the single valence bond pegs with the single central hole in each atom face. The double and triple bond assemblies with two and three hole-peg connections appear to be adequate. The inadequacy of the single bond hole-peg connection is the more evident because it is around such single bonds that the complex and simultaneous rotations of the molecules occur. Much important information concerning these intramolecular motions can be obtained by a study of the motions of the structural model.

A New Method of Linkage for Atomic Models

The difficulty associated with this feature of the models has been successfully removed with a device developed by the writer recently in Princeton. Models containing hundreds of atoms have been constructed and subjected to manipulative test by his student, Mr. Bernard Becker, with most interesting results in the field of complex polypeptide and other structures. For the single bond peg we have substituted snap-fasteners of the type familiar in dress-making. Into each face of an atom model carrying the one-hole single valence we have bored a one cm. hole and into each such hole have driven the female end of a 1 cm. snap fastener, to a depth below the atom face equal to the thickness of the male half of the snap fastener. Two atom faces thus provided can then be linked together in a firm linkage by soldering together, back to back, two male portions of the snap fastener and inserting the unit so constructed in the two female units in the two faces. Accurately constructed, these give a most satisfactory single valency bond. They permit a much greater freedom of rotation about the bond than is possible with the hole-peg device while the stability during manipulation of molecular models so constructed is of an entirely higher order of excellence. Long polypeptide chains have been subjected to elaborate studies of folding possibilities without any collapse of the structure and with complete freedom of single bond rotation. Our earlier efforts with the hole-peg assembly always led to disruption of the structure. So modified, the Fisher-Hirschfelder molecular models can be a much more faithful

picture of the dynamic entity that a molecule must be. Even with relatively simple structures such as the hydrocarbons, butane, pentane and hexane, the snap-fastener single valence permits a much more effective study of the steric relationships involved. Recent research has shown that the rotation around single bonds is not entirely unrestricted, as early theories of organic chemistry demanded. The new molecular models offer the possibility of studying the phenomena involved more intimately than with the types of model used hitherto.

The Structure of Fibrous Proteins

The X-ray evidence in the field of the proteins is most definite in the case of the fibrous proteins. With the silk protein, fibroin, and the hair protein, keratin, precise knowledge is available concerning certain details, while in others there is much that is still indefinite. The data on silk fibroin, which is one of the simplest proteins, are in best accord with the view that silk fiber consists of long filaments of fully stretched polypeptide chains lying parallel to the fiber axis. The repeating unit occurs every 3.5 Å units and the constituent peptides are mainly those of the amino-acids, glycine CH_2NH_2COOH and alanine $CH_3CHNH_2 \cdot COOH$. These are among the simplest of the amino-acids and, hence, the side chains, R, which appear at right angles to the plane of the polypeptide chain are H and CH_3

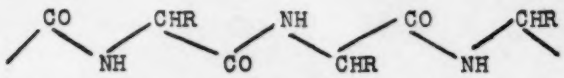

Fig. 5. The extended polypeptide chain.

respectively and therefore occupy relatively small areas along the chain.

Hair and wool protein or keratin possesses a property which distinguishes it sharply from silk fibroin, namely its large capacity to stretch and its elasticity. It is now known to exist in at least three forms. One of these, β-keratin, obtained by simple stretching of the hair, most closely resembles the silk fibroin. Its X-ray analysis suggests that it also is an extended chain of polypeptide units in which the side chains are more complex than in silk. The repeating unit in the "backbone" of β-keratin occurs every 3.33 Å units. Individual polypeptide chains are spaced 10 Å units apart which is the average distance required by side chains (which, however, show quite unequal lengths) normal to the backbone issuing from each chain. These side chains are both acidic and basic and may thus mutually saturate each other. Some of the connecting links between backbone chains are cystine molecules which account for the well known sulphur content of these proteins. In the β-keratin there is another spacing of about 4.5 Å units and this is regarded as the distance of separation in the third dimension of the polypeptide chains.

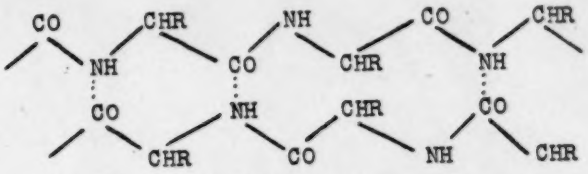

Fig. 6. Hydrogen bonded polypeptide chains.

(The R groups must be thought of as projecting alternately above and below the plane of the paper.)

It is assumed by many that successive chains in this dimension are held together by so-called hydrogen bonds N—H···O—C.

The stretched form or β-keratin is not, however, the normal form of hair keratin. Normal or α-keratin must be a folded structure. It is these folds which are drawn out when the fiber is stretched and the stretched condition may be "set" by exposure to steam heat while the tension is maintained. It is this process of "stretching" and "setting" which has attained the dimensions of a fine art, common to both sexes, in the "permanent wave" and "set" and in the "trousers crease". As is also well known to both sexes, this imposed change is only temporary, the hair or wool reverting gradually to its normal folded α-keratin structure.

The work of Astbury and his co-workers on α-keratin has revealed that this folded configuration may be derived from fully extended β-keratin peptide chains by assuming that the main chains contract to about one half of their original length, three peptide chains occupying 5.1 Å units or approximately one half of the 3×3.33 Å units in the β-structure. The side-chain spacing is maintained at about 10 Å units. The backbone spacing does not seem to be any longer present. Astbury has himself suggested a method of folding the stretched polypeptide chain to give a folded or buckled grid in which the chains take up hexagonal configurations. Others have sug-

gested that such folding into hexagons involves intra-molecular change leading to closed hexagons and this idea was systematically developed by Wrinch into her "cyclol hypothesis" of protein structure. Hydrogen bonding is an alternative method of producing such hexagon closing. A year ago Neurath examined such proposals with the aid of models devised by Mack and reached the general conclusion that "unless unreasonable distortion of bond angles are assumed, these configurations are too condensed to permit residues (side chains), other than those of glycine and alanine and hydrogen atoms to get into the positions called for by the carbon atoms of the hexagonal rings to which they are attached." He further concludes that "from consideration of space requirements of amino acid residues it follows also that the cyclol structure is too condensed to permit the accommodation of side chains." The cyclol hypothesis and, by implication, Astbury's first-proposed structure for α-keratin have recently been severely criticized by Pauling and Niemann on these and other grounds. Astbury has himself quite recently accepted the conclusions reached by Neurath in the matter of space required by the side chains.

Some indications of the manner in which these difficulties might be surmounted are contained in the final sentence of Neurath's paper, "The question suggests itself, therefore, whether a contracted form of polypeptide chains may not be arrived at by evoking a three-dimensional folding of the main chains which would still preserve the alternate orientation of the side chains." Astbury himself remarks, "We must consider seriously now whether we ought not to accept once and for all at least the conclusion that the intramolecular folds of the proteins are in essence always of the β-type. Something like that is what the X-ray findings have been pointing to for a long time." In the final section of his paper he indicates that a possible manner whereby this may be achieved is now under study and that the results while still incomplete are promising.

Molecular Models of Folded Polypeptides Chains

The stable molecular models of polypeptide chains made possible by the "snap-fastener" single bonding described in earlier paragraphs produce the necessary flexible yet stable systems with which alternative mechanisms of folding of β-chains may be studied. Mr. Bernard Becker has been able to show that there are types of folding which are at once compatible with the requirements of the X-ray data and still leave adequate spacings for the side-chain demands placed on such folded structures. Quite generally our experiments with the new flexible models reveal that we are as yet only at the beginnings of our knowledge of the manifold ways of folding such chains of polypeptides and that, in principle, there are many possible solutions.

The unit in a β-polypeptide chain is the sequence of groups CO—NH—CHR. This grouping, when the bond angles are properly located, is not far from a planar model. It is easy to show that such peptide units can be arranged in a continuous chain in which each unit is packed, back to back with other units, in what we may term the one up one down or filter-press configuration, as illustrated in Fig. 7. The three-

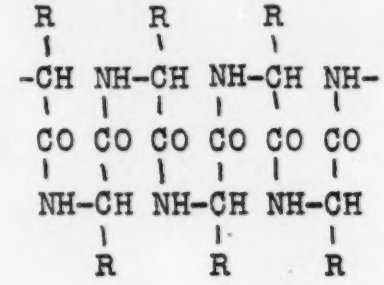

Fig. 7.

dimensional model of such a structure shows it to be possible and compact, with ample room for the side chains, R, which emerge at right-angles on alternate sides of the axis of the structure produced by the "filter-press type" arrangement. Measurement of this unit shows that it repeats every 4.5 Å units. This would not satisfy the dimensional requirements of α-keratin. It has, however, features that are both suggestive and instructive. It lends itself readily to double hydrogen bonding between adjacent chains.

We have already mentioned that attention has been directed to units of six peptides and that objections have already been raised to Astbury's suggested method of folding and the Wrinch "cyclol-6" unit. The type of folding just described can be bent to give a closed 6-peptide unit. A triangular prismatic unit is produced having quite definite interest. Inspection shows that there is a repeating triangular pattern in the

structure. The side-chain bonds on top form a small triangle, the CO groups a large triangle. On the bottom side the side-chain bonds form a large triangle and the CO groups a small triangle. Similar conditions obtain with the NH groupings. On top and bottom there is ample space for any conceivable array of side chains. By bringing such six-peptide units into conjunction with one another so disposed that adjacent units are, alternately, top meeting bottom and vice versa at the triangle corners, geometrical patterns of 6-peptide units can be produced. The configuration at the angles is such that CO in one is adjacent to NH in the next and vice versa. Such units then can tie together by the "hydrogen-bonding" association so frequently postulated in protein structures. It is easy to show, however, that the triangular pattern can be developed with a continuous —CO—NH—CHR— linking by crossing over at the angle of the triangles with a CO–NH linkage between groups in the two triangles as in Fig. 8. We have constructed

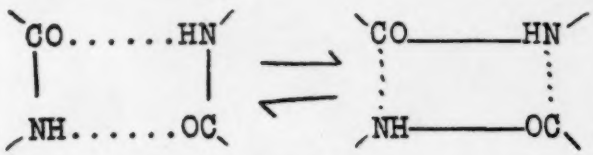

FIG. 8.

symmetrical arrangements of such triangular 6-peptides to give the 96-peptide unit. The next larger symmetrical unit of this pattern would contain the much discussed 288-peptide unit which is the estimated size of a number of globular proteins in the 35,000 molecular weight range. So long as such patterns are planar there is no difficulty as regards accessibility of and adequate space requirements for a normal sequence of side chains.

The 6-peptide structure just considered suggests at once that a 3-peptide can be equally well arranged in a closed unit. The side chains can be disposed in two ways either projecting outwards at 120° angles in the plane of the structure or vertically to that plane. Both of these structures can be arranged in geometrical patterns with the side chains as the connecting links in the former and hydrogen bonding or CO–NH linkages in the latter. In this latter case, cage structures may be produced with the side chains projecting vertically from the cage faces. Two such tripeptide closed structures placed back to back give a "ham-sandwich" structure with the NH groupings the "ham" between the two CO·CHR "bread" layers.

A Molecular Model for Normal Hair Fiber

What is apparently of greater significance, however, is that a 3-peptide fold of an indefinitely long peptide chain can yield a folded spiral. The three dimensional model thus obtained derives from the planar structure of the previous paragraph and has many highly satisfactory features. Thus, the keto-groupings arrange themselves in 3 linear arrays making 120° angles with the central axis of the fold. The same is true of the NH groupings and the side chain bondings. Inspection shows that, in the case of these latter, there is again ample room for normal side-chain sequences. Measurement shows that this particular method of fold gives accurately the spacing characteristics required by Astbury for the normal folded-hair or α-keratin structure. The repeating unit has quite accurately a 5.1 Å unit spacing. The side-chain distances as with β-keratin would average about 10 Å. Stretching this folded structure would give the 100 per cent extension demanded by the properties of hair-fiber and the result would be the β-keratin structure. It obviates the difficulty arising in connection with earlier proposals as to folding which required that all the R-groups of the side-chains be either "on the top or the bottom of the chains where there is insufficient room to accommodate them" (Bull). In our model Fig. 9(a) and 9(b), also, there is a favorable disposition of the side-chains to exercise that interaction with one another which must play a role in determining that keratin exists in the contracted α-form whereas silk fibroin whose side-chains are typically non-polar does not show the tendency to form the α-type fold. Our 3-peptide folded spiral therefore reproduces with great fidelity the demands of our present knowledge as to the α-keratin structure, including the disappearance of the 4.5 Å unit spacing. It represents, therefore, one possible solution for this structure. In the keratin group are included, in addition to the hair fibers, the protein of bird feathers, nails, claws and porcupine quills.

Other Alternative Foldings of Extended Hair Fiber

We have examined an alternative folding of the peptide chain which also yields the 5.1 Å

FIG. 9*a*. Spirally folded polypeptide chain.

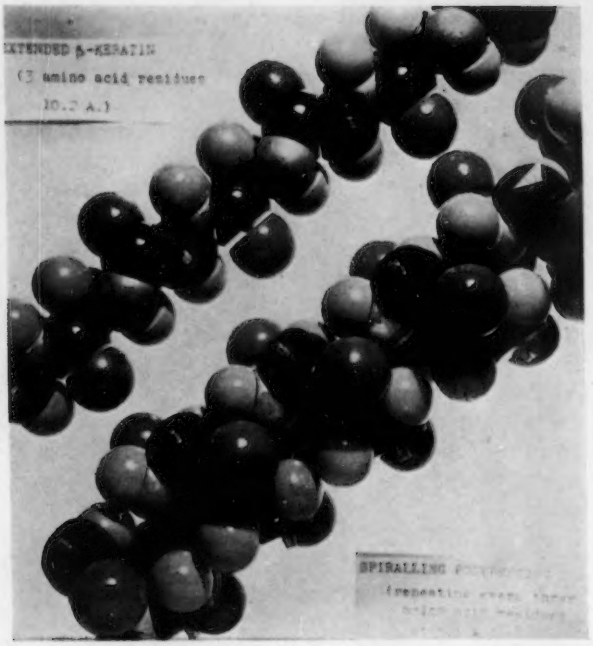

FIG. 9*b*. Comparison of spiral fold with extended chain.

The skeleton of a hair or wool fiber, seen through atomic spectacles, would appear somewhat like the model on the left, which represents, as printed, a magnification of about 50 million diameters. It is a spiral fold of units each containing the atom sequence —CO·NH·CHR. There are three such units in each turn of the spiral. The R-groups, known as the side chains, are not included in this photograph. They would be attached to the fiber at the positions shown by the bevelled pegs in the model. On subjecting this model to tension it will stretch to 100 per cent of its own length to give the stretched or extended form, as do hair and wool fibers. This stretching is assisted by heat and steam. It is hair so stretched which can be "permanently waved" or "set." The "trousers' crease" is also applied to the stretched wool fiber. Both the normal and the extended fibers are shown in the photograph on the right.

repeating unit. We have characterized this as the one up-two down fold illustrated graphically in Fig. 10. We do not regard such a structure, although free from major objections, to be so satisfactory as the one just considered since it causes a close association of two out of three side chains of a sequence along one face of the spiral and at 3.5 Å units apart. These positions would accommodate side chains which are not unduly large and permit larger side-chain units to be accommodated on the remaining side-chain position.

We are convinced from experiments with our flexible peptide chains that the conclusions of Neurath with respect to the non-spiral type of folding suggested by Astbury cannot be considered final. Our own trials have indicated the definite possibility of a planar folding with adequate side-chain spacing involving however a definite angular displacement from cis- and trans-folding at the single bonds in the chain.

As is well known, hair keratin exists in another state, the "super-contracted" form. It assumes this form after being stretched and treated with heat and moisture for a short time; if it is then allowed to contract, it will contract to a shorter length than the normal α-form. The X-ray analysis does not reveal any new definite repeating pattern for this form. Experiments with our flexible β-chains indicate that spiral folding can be achieved with units of 4, 5 and 6 peptides

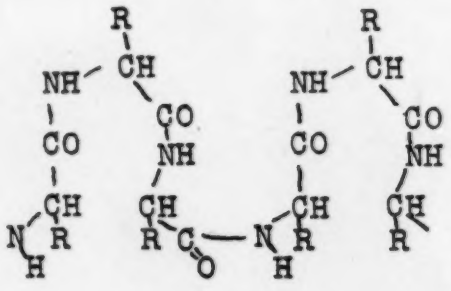

FIG. 10.

in a single spiral fold without any particular difficulty as to disposition of side chains save that the space available becomes progressively smaller. It is obvious that such spiralling would produce contracted forms of the keratin considerably shorter than the α-form. It would probably yield a 5.1 Å unit distance. There is no evidence, however, that would compel the formulation of such a spiral interpretation of the super-contracted state. There is in the judgment of certain workers some evidence that what is achieved in the production of super-contracted keratin is a randomly folded and randomly arranged keratin chain. Our folding experiments definitely indicate the possibility of this since we have examined many other patterns of folding than those already discussed in detail without noting insuperable difficulties. It is well known that the random folding of rubber from the stretched, crystalline form, to a normal form only 10 per cent the length of the stretched chain must involve a high degree of random folding.

Myosin and Collagen Fibers

The protein of muscle, myosin, as Astbury has emphasized, is found to have the extension and contraction characteristic of hair fiber, with, according to Astbury, the α-configuration as the normal resting state, the super-contracted form as the contracted tissue and a possible extension to the β-form with tension. In muscle tissue the contraction and return to the normal state are chemically induced. The absence of cystine linkages in myosin probably facilitates the attainment of the super-contracted state whereas these "vulcanizing" linkages in keratin tend to prevent this in hair. In the collagen-group fibers of connective tissue the presence of proline as an important constituent amino acid modifies the attainable extension. We have shown that proline can be built into such amino-acid chains. As Astbury has suggested, the presence of a partially cis-configuration in the fold may account for a smaller peptide dimension that has been postulated in this case.

It is apparent, from the complexities of the problem in the case of the fibrous proteins already outlined, that the problem of the globular proteins must be essentially one of a higher order of complexity. We will content ourselves, therefore, with the observation that it is possible that there is no single unique solution of the globular protein problem, that the ease with which the polypeptide chain can fold into units of 2, 3, 4, 5, 6 and even higher numbered folds offers the possibility of very many different solutions. Progress to finality will involve the exploration of many pathways of inquiry.

Molecular Models of Synthetic Structures

The examination of the polypeptide chain in the production of protein structures represents one important section of the problems that can be examined with the new molecular models. Preliminary excursions into other fields have already been made. They are instructive in the field of chemo-therapy. We have photographed, among others, models of sulfanilamide and sulfapyridine. The hormone, thyroxin, is an interesting structure. Among the vitamins, vitamins A, B_2, B_6, nicotinic and pantothenic acids, vitamin E and K_1 make interesting models. In the sterol group, models of cholesterol, cholestanol and coprostanol serve to check data on molecular dimensions of these large molecules. In the models of carcinogenic substances of the benzanthracene and benzphenanthrene types there appears to be some correlation between activity and planar and non-planar configuration of the molecules. In the field of three-dimensional synthetic polymers of the plastic field the molecular models are especially useful in indicating structural arrangements. Polystyrene and lucite polymers are illustrative of these.

Paralleling the protein field there is a similar area of study in the carbohydrate field. The polyoxymethylene and polymethylenes give fibrous structures. The cellobiose unit builds up into the structures of cotton and wood cellulose and the gluco-pyranose group into the starch molecule. The nature of the repeating units and of the linkage between them is now under active study. Synthetic fibers are derived from cellulose in acetate and viscose rayons. More recently nylon takes its place as a synthetic linear polymer prepared by condensation of a diamine and a dibasic acid condensed into a chain of indefinite length. In the field of natural and synthetic rubbers there are analogies to the protein problem that we have discussed in detail. The stretched rubbers are crystalline and are

known to be extended chains. On release of tension these chains fold to give the normal state of the substance. Experiment shows that folding similar to that in the polypeptides is easily possible, but the final structure undoubtedly contains an extensive randomness of fold showing no X-ray pattern. In rubber, also, the process of vulcanization establishes cross linkages between the hydrocarbon chains and thereby decreases the natural characteristics of the rubber molecule. The exploration of any given field brings a multiple reward in the increasing knowledge gained not only in the field investigated but in all the other parallel fields of study.

ACKNOWLEDGMENT

A final word of indebtedness to the author's pupil, a Princeton Senior, Mr. Bernard Becker, is here expressed. Of him it may be said, to adapt the lines of Francis Thompson in "Her Portrait", "And teaching him, by his ingenious art, the master threefold learns for all he can impart." Mr. Becker has learned to manipulate the atomic models here discussed so skillfully that they have revealed to both of us very much more than either of us could ever have anticipated and have provided us with the equivalent of more efficient atomic spectacles with which to observe large molecules.

CRYSTALLINE DIPHTHERIA ANTITOXIN[1]

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(*Read April 24, 1941*)

ABSTRACT

Diphtheria toxin forms a precipitate when mixed in certain proportions with serum from a horse which has been immunized against the toxin. This precipitate is a compound of the toxin and antitoxin. The compound is dissolved in slightly acid solution and the toxin digested by trypsin. The antitoxin remains in solution. About 30–60 per cent of the original antitoxin may be recovered in this way. This "crude antitoxin" may be further separated into three fractions by precipitation with ammonium sulfate at about pH 7.2.

The fraction soluble in 0.35 saturated ammonium sulfate but insoluble in 0.65 saturated ammonium sulfate has a high antitoxic titer (700,000–1,000,000) antitoxin units per gram protein nitrogen; 90 per cent or more of the protein is precipitated by diphtheria toxin. It is strictly homogeneous in the ultracentrifuge with a sedimentation constant of 5.7×10^{-13}. It shows only one boundary at pH 7.3 in the electrophoresis cell but there is some reversible spreading. This fraction corresponds probably to the highly purified preparations of Pope and of Pappenheimer and their collaborators.

Solubility determinations show that this fraction is still quite inhomogeneous. Extraction of this fraction with 0.5 saturated ammonium sulfate at pH 7.2 yields a small amount of a protein which has constant solubility and is therefore probably a pure protein. This protein crystallizes readily in the form of poorly formed thin plates. The antitoxic value is unchanged after three recrystallizations.

VANBEHRING and Kitasato in 1890 found that the sera of animals which had received repeated small doses of diphtheria toxin developed the property of neutralizing the poisonous effects of the toxin. This neutralizing substance was called antitoxin. Diphtheria antitoxin was the first such substance discovered and is still one of the most powerful therapeutic agents known. Further study of diphtheria and other antitoxins has shown that they are proteins closely related to the normal serum proteins. They must possess some characteristic chemical structure, however, not present in the normal proteins since they neutralize toxin whereas normal serum proteins do not. Knowledge of the structure responsible for the antitoxic power may eventually lead to the synthesis of antitoxins of greater therapeutic value. The first step in the attempt to identify the structure responsible for this therapeutic value is the isolation of the antitoxin in pure form. Preparations of pneumococcus antibody, which are pure in the sense that they are completely precipitated by antigen, have been obtained by Chow and Goebel (1935), Chow and Wu (1937) Heidelberger and Kendall (1936) and their coworkers. Petermann and Pappenheimer (1940) prepared diphtheria antitoxin which was homogeneous by electrophoresis or ultracentrifuge but was not completely precipitated by toxin. Highly purified preparations of diphtheria antitoxin have also been obtained by Pope and Healey (1939) and coworkers.

Ramon (1922) showed that under certain conditions diphtheria toxin and antitoxin combine to form an insoluble precipitate. Evidently recovery of the antitoxin from this precipitate would result in a very considerable purification. Ramon was able to recover some antitoxin from this precipitate by heating in slightly acid solution (Ramon, 1923). Pope (1939) found that most of the antitoxin could be recovered from such precipitates by treatment with pepsin in slightly acid solution and the work has been confirmed and extended by Pappenheimer and coworkers. Pepsin destroys both toxin and antitoxin to some extent and it seemed possible that better yields might be obtained by using trypsin. It was found that trypsin has no effect on the toxin-antitoxin in neutral solution but if the complex is dissolved in dilute acid and then brought back to neutral solution the toxin is digested and 30–60 per cent of the antitoxin may be recovered.

It was thought at first that this result was due to dissociation of the toxin-antitoxin complex in acid followed by digestion of the toxin. It appears, however, that this is probably not the case since diphtheria toxin is very unstable in acid (pH 3.5) as Pappenheimer (1937, 1938) showed, and would be inactivated and precipitated were it present in solution. This does not occur when the toxin-antitoxin complex is al-

[1] The experimental results will be described in a forthcoming number of the *J. Gen. Physiol.*

TABLE I

OUTLINE OF A METHOD OF PREPARATION OF CRYSTALLINE DIPHTHERIA ANTITOXIN

33 liters toxin + 4 liters antidiphtheria plasma, 24 hours 20° C. Filter.	2×10^6 L_f	20 L_f/PN
Filtrate ← ↓ Precipitate		
Suspend in 5 liters M/10 KH_2PO_4 titrate to pH 3.7, add 2 gm crystalline trypsin 25° C. 20 hours. Titrate to pH 7.2 25° C. 24 hours. Filter.		
Precipitate ← ↓ Filtrate	6×10^5 L_f	350 L_f/PN
Adjust to 0.33 sat. ammonium sulfate and filter.		
Precipitate ← ↓ Filtrate	4×10^5 L_f	500 L_f/PN
Adjust to 0.45 sat. ammonium sulfate and filter.		
Precipitate ← ↓ Filtrate	2×10^5 L_f	$\frac{700}{800}$ L_f/PN
Adjust to 0.65 sat. ammonium sulfate and filter.		
Filtrate ← ↓ Precipitate		
Stir + 3 liters (0.5 sat. ammonium sulfate, 0.05 M pH 7.4 phosphate). Filter.		
Precipitate ← ↓ Filtrate	1.5×10^5 L_f	$\frac{700}{800}$ L_f/PN
Titrate to pH 3.5. Filter.		
Precipitate ← ↓ Filtrate	1×10^5 L_f	$\frac{700}{800}$ L_f/PN
Titrate to pH 7.0, add sat. ammonium sulfate slowly till very slight silky precipitate forms. Stand 6° C. 24 hours, centrifuge.		
Supernatant ← ↓ Precipitate		
Fine plates, needles and some amorphous. Suspend in 50 ml 0.5 sat. ammonium sulfate, add water till clear, add sat. ammonium sulfate till slightly cloudy, 25° C. 2 hours. Irregular plates, no amorphous. Centrifuge.		
Supernatant ← ↓ Precipitate	6×10^3 L_f	$\frac{700}{800}$ L_f/PN
Dissolve in 5 ml water and sat. ammonium sulfate slowly till slight turbidity. 25° C. 1 hour. Thin plates.		

lowed to stand in acid. The action of trypsin does not take place in acid but during and immediately after neutralization. It seems probable that the toxin-antitoxin complex dissolves as such in acid and the toxin is then split off by the trypsin during and after neutralization while the complex is still dissolved. If the toxin-antitoxin complex is allowed to precipitate again in neutral solution before the addition of trypsin, the toxin is no longer digested.

The "crude antitoxin" recovered after removal of the toxin contains 30–60 per cent of the original total antitoxin.

The preparation is 95 per cent or more precipitated by toxin and has an antitoxin titer of 300–400 units[2] per mg protein nitrogen. This preparation may be further purified by precipitation with ammonium sulfate (Table I). The fraction precipitating between 0 and 35 per cent saturated ammonium sulfate has an antitoxic value of about 550 units per mg protein nitrogen while that precipitating between 0.45 and 0.65 saturated ammonium sulfate has an antitoxic value of 700–800 units per mg protein nitrogen by the flocculation test.

The antitoxin value of this purified antitoxin, as determined by animal protection tests, has been determined by Dr. W. E. Bunney at E. R. Squibb and Sons, New Brunswick, N. J. Two preparations were tested and found to contain about 700 antitoxin units per mg protein nitrogen.

[2] One antitoxic unit is defined as the equivalent of a "standard antitoxin unit" and is approximately equivalent to the quantity of antitoxin which will neutralize 100 times the quantity of toxin required to kill a guinea pig in 5 days.

This fraction probably corresponds to a similar preparation obtained by Pope and Pappenheimer by means of pepsin digestion. It is homogeneous by electrophoresis and ultracentrifuge measurements. The solubility of this fraction, however, is not constant but varies with the quantity of solid present showing that it consists of two or more proteins which are indistinguishable by electrophoresis, rate of sedimentation or precipitation by toxin.[3] The fact that the solubility varies with the amount of solid present shows that the solid phase in the dilute suspension is different from that in the more concentrated. A partial separation of the system has therefore been accomplished and, on paper at least, all that is necessary is to repeat the process until no difference in solubility is observed. This is the theoretical basis for the fact that fractional precipitation has been of such great practical value in the purification of proteins.

In practice it is not always possible to complete the separation in this way owing to the fact that insufficient material is available or because the protein is too unstable and changes during the experiment. In the present case both difficulties are encountered. Nevertheless, it is possible to obtain a much more homogeneous protein by removing only the most soluble portion of the preparation. This is best done by extracting the solid precipitate with half saturated ammonium sulfate at pH 7.2. The protein obtained in this way is much more nearly homogeneous but still does not have constant solubility. Repetition of the extraction with this fraction would be expected to result in further improvement but the yield is so small that this cannot be done in practice. If this saturated solution is titrated to pH 3.5, however, a precipitate appears. After removal of this precipitate the protein has constant or nearly constant solubility and crystallizes readily in rather poorly formed thin plates (Fig. 1). These plates are slightly double refractile. They are slightly less soluble than the amorphous protein and their appearance in solution is greatly accelerated by inoculation of a supersaturated solution with some of the plates. They resemble very closely the first crystals of ribonuclease obtained by Kunitz. Ribonuclease crystals, however, become beautifully formed on recrystallization or on long standing while the antitoxin crystals do not improve and may become less well formed. This difference is due to the fact that the antitoxin is very unstable. Even after 24 hours at 20° C. some less soluble protein has been formed and after a few days the solubility curve of the preparation resembles that of the crude fraction from which it was derived. At the same time the flocculation time, when the antitoxin is mixed with toxin, increases although the final endpoint remains the same. The formation of this less soluble protein interferes with crystallization and soon prevents it altogether.

Similar changes in the rate of flocculation with aging have been noted by Glenny and others with antitoxic sera. It is possible that the pure antitoxin represents the protein in its original form and that the more insoluble proteins are derived from this unstable protein. So far no conditions have been found which prevent these changes.

Properties of the Antitoxin.—Purity. The electrophoretic pattern has been determined by Dr. A. Rothen. There is only one boundary but there is considerable "reversible spreading" in dilute phosphate buffer at pH 7.3. A possible cause of this phenomenon, which has been noted with several proteins, is the migration of water in the cell due to electroendosmosis. If this were the cause the spreading should not be observed under conditions which prevent the electroendosmosis. The experiment was therefore repeated in the presence of calcium chloride at pH 7.3 and at pH 3.0. Glass has no charge when in contact with these solvents and hence there is no electroendosmosis. There is no reversible spreading of the protein under these conditions.

The protein is strictly homogeneous in the ultracentrifuge with a sedimentation constant of $S_{20}^{water} = 5.3 \times 10^{-13}$. The diffusion coefficient is $D^{20} = 5.56 \times 10^{-7}$ cm²/sec and the molecular weight calculated from these figures is 90,500

[3] This result is simply another example of the fact that the solubility method, which is theoretically identical with the classical melting point method, will distinguish between closely related proteins which are indistinguishable by other methods. Thus Landsteiner and Heidelberger found that hemoglobin from donkey and horse could be distinguished by this method although serologically they are extremely similar. Crystalline pepsin (Herriott, Desreux and Northrop, 1940) prepared without special purification, is strictly homogeneous by electrophoresis or ultracentrifugation but does not have constant solubility and may be shown to contain several different proteins. Mixtures of samples of chymotrypsinogen of different solubilities are indistinguishable by electrophoresis (Butler, 1940) as are mixtures of pepsin and iodinated pepsin (Herriott, 1941) although in the latter case the solubilities are very different. Egg albumin from closely related species is also indistinguishable by the electrophoresis technique although they may be distinguished by serological tests (Landsteiner, Longsworth and van der Scheer, 1938).

FIG. 1. Crystalline diphtheria antitoxin.

(Rothen, in press). This is close to the figure obtained by Pappenheimer for a sample of antibody obtained by the action of pepsin. Purified antibody prepared by heating the toxin-antitoxin complex in acid has a sedimentation constant of 6.8×10^{-13}. Apparently, therefore, trypsin hydrolyzes part of the antitoxin molecule as well as the toxin. This agrees with Pappenheimer's experiments with pepsin.

The most highly purified fraction has nearly constant solubility. All the preparations of this fraction give better solubility curves than most proteins but only one shows really constant solubility. These curves, however, were all made on amorphous preparations and hence are open to some uncertainty as it is not possible to be sure that they represent equilibrium values. The crystalline preparations do not give as good curves owing to the fact that some less soluble material is formed while the crystallization is taking place.

The protein contains about 2.5 per cent carbohydrates determined as glucose by Sorenson's method.

Precipitation of Purified Antitoxin and Toxin

Solutions of various samples of purified antitoxin were mixed with an equivalent of crude toxin, obtained from E. R. Squibb and Company, or of purified toxin. The solutions were kept at 50° C. for 20 hours, centrifuged, and protein nitrogen determined in the supernatant. 4 to 10 per cent of the total protein nitrogen present remains in the supernatant with both the pure antibody having an antitoxic 800 L_f/mg P.N. and with the fraction precipitated between 0.35–0.45 saturated ammonium sulfate (400–500 L_f/mg P.N.) and with either crude or purified toxin. 90–95 per cent of the total protein nitrogen, therefore, precipitates out. It is not possible to say at present whether the protein nitrogen which fails to precipitate comes from the toxin or antitoxin.

This result indicates the presence of at least 2 antitoxins which combine with different amounts of toxin. Evidence for the existence of 2 such antitoxins has been obtained by Kekwick and Record (1941).

Precipitation Zone with Crude and Pure Antitoxin and Toxin

Mixtures of immune serum and crude toxin precipitate only over a range of concentration in which the two substances are present in the proportion of less than 2 to 1; that is, if complete flocculation occurs with a 1 to 1 mixture the precipitation will be incomplete if more than 2 equivalents of either component is present.

With pure antibody and crude toxin the range is about doubled and precipitation occurs over a range of 4 to 1.

With pure antibody and purified toxin (kindly supplied by Dr. A. M. Pappenheimer, Jr.) the range of complete precipitation is very wide and the components may be varied in the ratio of at least 64 to 1.

Immunological Reactions

Some of the immunological properties of the purified toxin have been determined by Dr. Carl Ten Broeck. The serum of a rabbit immunized against normal horse serum gave a precipitate with 1/4,000 ml normal horse serum (containing about 0.002 mg protein nitrogen) but gave no precipitate with 1 ml of a solution of purified antibody containing 1/10 mg protein nitrogen.

Guinea pigs sensitized with purified antibody reacted on injection of 0.05 mg antibody protein nitrogen, whereas 5 mg normal horse serum protein nitrogen was required to cause a reaction.

The purified antitoxin is, therefore, antigenically distinct from the normal serum proteins.

The toxin and the anti-diphtheria horse plasma used in this work were obtained from the Biological Laboratory of E. R. Squibb and Sons, New Brunswick, N. J.

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THE METHOD OF ACTIVATION OF MELANOPHORES AND THE LIMITATIONS OF MELANOPHORE RESPONSES IN THE CATFISH AMEIURUS

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ABSTRACT

In the skin of a pale catfish the large melanophores are represented by black spheres some 45 microns in diameter. In a fully dark catfish each such melanophore covers an area with a diameter of some 145 microns. The concentrated state of the melanophores is induced through nerve fibers that presumably discharge adrenaline (adrenergic fibers). The dispersed state is induced through either nerve fibers that probably discharge acetylcholine (cholinergic fibers) or through intermedine carried from the pituitary gland of the brain by the blood to the melanophores. If the pituitary gland is removed from a catfish, this fish can still darken but only partly (each melanophore can disperse its pigment to cover an area the diameter of which is at most about 100 microns). This reaction must depend exclusively upon cholinergic fibers (acetylcholine). When additional acetylcholine is injected into such a fish, the melanophore pigment is not further dispersed. This is true even when enough acetylcholine is injected to kill the fish. If into such a partly darkened fish a small amount of intermedine is injected, the fish will then fully darken. Intermedine, if injected into a pale fish, will also completely darken it. Hence acetylcholine as compared with intermedine is capable of inducing only a limited and well defined dispersion of pigment in catfish melanophores. The blanching agent adrenaline shows no such limitation in its action. It is noteworthy that acetylcholine in large doses is deadly to catfishes, but intermedine is not. What restricts the action of acetylcholine on the melanophores of this fish is not known. When under natural conditions a fish darkens, its melanophores are first excited through nerves (acetylcholine) and only subsequently does intermedine become an effective agent.

1. INTRODUCTION

WITHIN the last few years much that is new has been learned about the color changes in catfishes. It is now generally recognized that these changes are dependent almost exclusively upon melanophores which are of two kinds, micromelanophores in the epidermis, and macromelanophores in the derma. Of these the macromelanophores are the more efficient notwithstanding the fact that the layer in which the micromelanophores are located may become heavily impregnated with melanin particularly in fishes that have been for some time in the dark phase (Odiorne, 1937). It is also commonly understood that the parts chiefly concerned with chromatic activation in *Ameiurus* are three: the pituitary gland, and two sets of nerves one dispersing and the other concentrating (Parker, 1934*a*). The pituitary gland acts on the melanophores through its product intermedine, carried in the blood and lymph from the gland to the color cells; the dispersing nerve-fibers are effective through their special neurohumor acetylcholine; and the concentrating fibers through adrenaline. These two sets of nerve-fibers have been classed in consequence of the neurohumors associated with them as cholinergic and adrenergic respectively (Parker, 1941).

Abramowitz (1936) appears to have been the first to point out that when two kinds of catfishes, one hypophysectomized and the other normal, are placed in a black-walled, illuminated vessel, the hypophysectomized individuals after adjustment of color are never so dark as the normal ones. This condition was independently noted by Veil (1937). But it remained for Osborn (1938) to show in a quantitative way what this limitation was. According to Osborn a normal catfish can disperse the pigment of its macromelanophores so that the color cells will cover areas the diameters of which average some 125 microns whereas a hypophysectomized individual can disperse this pigment only to some 70 microns. Osborn's statements were confirmed by Wykes (1938) on *Ameiurus* and by Enami (1939) on *Parasilurus*. Parker (1939*a*), who worked on *Ameiurus* as Osborn and Wykes had done, placed the diameter of the macromelanophores in a normal, fully dark catfish at some 145 microns and in a hypophysectomized one at about 100 microns. The differences between the estimates of Osborn and of Parker appear to be due to the fact that Osborn based his averages on small as well as on large macromelanophores whereas Parker measured only the larger and presumably mature color cells of this class. It is, however, perfectly evident from these several

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records that hypophysectomized fishes fail to disperse their melanophore pigment as fully as normal fishes do. It is the aim of this paper to discuss the process of melanophore activation in *Ameiurus* as well as the limitations of the pigment activities in this fish.

2. Materials

The fish used in this work was the common New England catfish *Ameiurus nebulosus*. For the sake of uniformity tests were restricted to specimens about 15 cm. in length. Such fishes weighed very closely to 50 grams each. When operations on living fishes were necessary the animals were first anesthetized by the convenient method of immersion in ice-cold water (Parker, 1939*b*). Much of the hypophysectomized material used in this work was prepared for me by Dr. L. H. Kleinholz to whom I wish to express my thanks. I am also under obligations to Mr. Frank White for the illustrative photographs.

The range of color change in the catfish is shown in Fig. 1 where individuals from the palest to the darkest are shown. Figs. 2 to 7 illustrate the states of the melanophores in three of the five stages seen in Fig. 1. The macromelanophores and micromelanophores of the palest stage are shown in Figs. 2 and 3 respectively. In both instances the pigment masses are approximately spherical, the diameter of the sphere in the macromelanophores being some 45 microns, that of the micromelanophores some 12 microns. The macromelanophores of the fully dark or coal-black stage are shown in Fig. 6, the micromelanophores in Fig. 7. The pigment in the macromelanophores of a coal-black fish covers incompletely an irregular area about 145 microns in diameter. The micromelanophores at this stage have irregular outlines with a few relatively long processes. These processes in many instances either anastomose or overlap in such a way as to appear to unite. A third and important color stage in the catfish is that seen in the middle fish in Fig. 1. This may be called the intermediate stage. At this stage the macromelanophores have short stubby branches (Fig. 4) and cover as a whole an area the diameter of which is roughly 100 microns or less. The micromelanophores of the intermediate stage (Fig. 5) are irregular in outline and possess relatively few rather delicate processes. These are apparently never in contact one with another.

3. How Neurohumors Act on the Melanophores in Ameiurus

From the time of Spaeth's work on the color cells in fishes (1913) it has been customary to regard chromatophores the nerves of which have been severed as free from all forms of nervous control and in that sense independent. But it must be remembered that chromatophores in newly denervated areas of skin or in scales taken freshly from the fish's body still have attached to them more or less of the original nerve with its terminals. Agents which under these circumstances activate such color cells may do so either indirectly through the remnant of nerve still attached to the cell or directly by immediate contact with the protoplasm of the color cell itself. Thus this technique can yield no conclusive answer to the question of the indirect or direct action of such agents on color cells. This defect, which runs through almost all of the earlier work, was pointed out some years ago (Parker, 1934*b*) and it was then proposed to use in such investigations only those fishes which had been kept alive long enough after the severence of their chromatic nerves to insure the complete degeneration of these nerves. It was found by appropriate trials that ten to twelve days was ample time for this purpose. After the expiration of such a period no evidence of normal nervous activity could be discovered in the chromatic nerves and it was assumed that the necessary degree of degeneration had been reached. Chromatophores in such denervated areas were believed therefore to be completely free of all possible nervous associations. In inducing this degree of nerve degeneration it was found necessary to keep the fishes in water at about 20° C., for at the usual winter temperatures of 6° to 8° C. degenerative changes went on only very slowly or even not at all.

To prepare the melanophores in catfishes for tests with neurohumors the fishes were first hypophysectomized to rid them of their natural source of intermedine. Immediately after this operation two rather distantly related rays in their tails were cut whereby two caudal bands were produced. Such fishes were then set aside for from ten to twelve days to allow time for the disappearance of the last traces of intermedine from their blood and for the degeneration of the chromatic nerves in their caudal bands. The fishes were kept in an illuminated vessel the walls of which were of a grayish tint with the result

that each fish with its caudal bands assumed sooner or later an intermediate coloration. Once or twice during this preparatory period the incisions by which the caudal bands had been originally formed were recut to prevent the possibility of nerve regeneration. At the conclusion of this period it was assumed that the fishes were ready for neurohumoral tests in that they had lost all traces of intermedine and their caudal bands were devoid of active nerve-fibers. Catfishes in this state were then subjected to tests with the three neurohumors, adrenaline, intermedine, and acetylcholine.

The adrenaline used in these tests was the commercial preparation made by Parke, Davis and Company. It contained one part of adrenaline chloride in 1000 parts of fluid which in the main was physiological salt solution. In previous studies (Parker, 1940) of the action of adrenaline on the melanophores of *Ameiurus*, fishes of 50 grams weight were injected each with 0.2 cc. of this neurohumor at different strengths. The solutions varied from 1 part of adrenaline in 5000 parts of Ringer's solution to 1 part in 40,000,000 of the solvent. Solutions of 1 part of the neurohumor in 5000 parts of solvent to 1 part in 1,000,000 of solvent induced a general blanching of the whole catfish; 1 part of adrenaline in 5,000,000 or 10,000,000 parts of Ringer's solution caused a pale spot near the region of injection; and 1 part of adrenaline in 20,000,000 or 40,000,000 parts of solvent was without effect on the color of the fish. These figures agree fairly well with those given by Bray (1918) as well as the more recent ones by Pierce (1941).

In testing the way in which adrenaline acts on the melanophores by the catfish three hypophysectomized fishes with caudal bands prepared as already described were injected each with 0.2 cc. of adrenaline 1 part in 5000 of Ringer's solution. In 10 minutes the fishes had blanched fully though the caudal bands were not always as pale as the rest of the tail. In about an hour, however, the bands had become as fully pale as the rest of the fish and remained so for some two to three hours. In a second set of prepared catfishes an injection of 0.2 cc. of adrenaline, 1 part in 5000 of solvent, was followed in a quarter of an hour by moderate blanching of both bodies and bands which was intensified by a second injection of adrenaline. In these instances both bodies and bands blanched at essentially the same rate. Since the melanophores in these bands were fully devoid of nerve fragments and yet responded to the adrenaline by concentrating their pigment it seems reasonable to conclude that this neurohumor acts directly on these color cells. This conclusion had already been reached in an earlier series of tests with this reagent on the color cells in the killifish *Fundulus* (Parker, 1934*b*).

The second neurohumor to be tried on the melanophores of the catfish was intermedine. This material was obtained by extracting with Ringer's solution the fresh pituitary glands of frogs or of catfishes or more conveniently by using Parke, Davis and Company's preparation of obstetrical pituitrine which contains intermedine as a byproduct. In the commercial manufacture of this preparation a small amount of chloretone is regularly added, a drug which is known to induce pigment dispersion in melanophores. However the amount present in this preparation, 0.5 per cent, was found to be quite ineffective in altering the states of the melanophores in *Ameiurus*.

Two catfishes of intermediate tint and prepared by hypophysectomy and caudal band formation were injected each with 0.2 cc. of obstetrical pituitrin (2 units) and allowed to remain for six hours in an illuminated vessel with gray walls. During this interval no color change either on the body of the fish or in its caudal bands was observed. Other catfishes prepared as those just described were injected with two doses each of 0.2 cc. pituitrin half an hour apart. These very regularly darkened slightly, the caudal band deepening in tint with the rest of the body. In some fishes a third and in others even a fourth dose of pituitrin was administered, whereupon the bodies of the fishes and their caudal bands often became very dark and in two instances the individuals were fully coal-black including their caudal bands. In other fishes two in number the dosage of pituitrin was double that just mentioned, namely 0.4 cc. per injection. These fishes also darkened and did so with this initial dose. In these instances as with the others the caudal bands and bodies darkened together.

An extract in Ringer's solution made from a single pituitary gland from a frog when injected into a prepared catfish profoundly darkened both body and caudal bands. The same was true of an extract of the pituitary gland of the catfish itself though this extract appeared to be less effective than that from the frog. Since in the presence of intermedine the melanophores of the caudal bands in prepared catfishes disperse their

pigment even to the maximum degree it seems fair to conclude that this neurohumor acts directly on these color cells without involving any special intermediate mechanism such as dispersing nerves.

The third and last neurohumor to be used in these tests was acetylcholine. This like intermedine is a darkening agent. It was used in the form of the commercial chloride. As is well known this agent is rapidly destroyed when injected into the animal body by the cholinesterase of the tissues. To prevent this destruction a preliminary dose of eserine is commonly given to the experimental animal, one part of eserine to 50,000 parts by weight of animal. At this concentration, however, eserine will darken a catfish more or less, but by reducing the amount to one part of eserine in 200,000 parts by weight of fish a concentration can be arrived at which will not darken the fish and will still protect the acetylcholine. This concentration was used in the following tests.

Into palish catfishes previously prepared by hypophysectomy, caudal band cutting, and subsequent injection with eserine, 0.2 cc. of solutions of acetylcholine of different concentrations from 1 part of the reagent in 100 parts of Ringer's solution to 1 part in 100,000,000,000 parts of solvent were injected. Nine different concentrations were used and all mildly darkened the catfishes except the weakest, 1 part of acetylcholine in 100,000,000,000 parts of Ringer's solution, which was without effect on the color of the fish. Three fishes in all received injections of this weakest solution and none of them showed signs of darkening. When two of these fishes were subsequently injected each with 0.2 cc. of acetylcholine 1 part in 100,000 of solvent they darkened moderately in half an hour showing that they were susceptible to the neurohumor. Concentrations of 1 part of acetylcholine in 100 parts of Ringer's solution to 1 part in 1000 of solvent not only darkened catfishes but led soon after to their death. The range of concentrations of acetylcholine within which this neurohumor was satisfactory for physiological tests was from 1 part in 10,000 to 1 part in 1,000,000,000.

The darkening that resulted from these various concentrations of acetylcholine made its appearance slowly and usually required from 20 minutes to half an hour to reach its complete development. It is a matter of interest to ascertain the relation of these physiologically effective concentrations to that found in the skin of a catfish which had darkened normally. The concentration of acetylcholine in the skin of such a catfish has been estimated to be 1 part of the neurohumor by weight to 13,000,000 parts of skin (Parker, 1940). How is this concentration related to the range of the physiologically active concentrations that have just been given? When 0.2 cc. of a given concentration of acetylcholine is injected into a fish of 50 grams weight this neurohumor must be diluted thereby to 1/250 of its original strength. Consequently to produce in the fish as a whole, including its skin, a mixture of 1 part of acetylcholine in 13,000,000 parts of fish it would be necessary to use for injection a solution containing 1 part of acetylcholine in 52,000 parts of solvent. This concentration lies well between the physiological extremes already given for the catfish, namely, 1 part in 10,000 and 1 part in 1,000,000,000. That this concentration may well reproduce in the fish the state necessary to normal darkening is seen in the fact that when 0.2 cc. of such a solution are injected into a catfish of 50 grams weight the fish darkens in an entirely regular manner.

In the several tests with acetylcholine where the tails and caudal bands of pale, prepared catfishes were closely watched as they darkened, these two areas took on a deep tint together as was true when intermedine was similarly used. Hence it is concluded that acetylcholine like intermedine acts directly upon catfish melanophores and without the intervention of any additional elements. Thus all three of the neurohumors tested in this investigation, adrenaline, intermedine, and acetylcholine, appear to act directly upon the melanophores of the catfish in that their solutions activate a given color cell whenever there is mutual contact.

4. Limitations in the Effects of Neurohumors

When 0.2 cc. of a solution of adrenaline, 1 part in 50,000, are injected into a catfish the whole fish irrespective of its original tint will blanch completely and under such circumstances the creature may well reach a maximum paleness (Fig. 1, extreme left specimen). Its macro- and micromelanophores will be found to be reduced to the spherical form (Figs. 2, 3). Thus adrenaline when fairly concentrated acts with complete efficiency throughout the whole range of melanophore response and induces full pigment concentration. When a weak solution of adrenaline, 1

part in 5,000,000 for instance, is injected subcutaneously into a catfish of intermediate tint, a pale area develops around the region of injection but this paleness does not spread far out over the skin. If the edge of this pale area is examined under the microscope, all transitions in melanophore states will be found there between those with fully concentrated pigment and those with pigment of an intermediate concentration. In other words this edge is not made up of a mixture of melanophores all of which have their pigment either fully concentrated or largely dispersed. This fact, that all intermediate degrees of spread of pigment are observable in this area, indicates that the "all or none law" appears to have no application to the activity of these melanophores. They seem to respond to varying degrees of stimulation and come to rest at any appropriate point. From this standpoint melanophores resemble smooth-muscle fibers (Spaeth, 1916).

In one respect the blanching of catfishes presents a slight anomaly. Many catfishes when they become pale in illuminated, white vessels fail to reach full paleness; they stop a little short of this end point. One of these is shown in the second fish from the left in Fig. 1. Under the microscope the melanophores in such a fish are not so nearly spherical as those shown in Figs. 2 and 3, but exhibit commonly slight protuberances, the roots of the pigmented processes. This state of incomplete blanching has already been recorded (Parker, 1940) and its common occurrence in hypophysectomized animals has led to the suspicion that in addition to adrenaline another concentrating neurohumor may be present in the catfish, one which can induce only incomplete blanching. I have, however, no conclusive evidence for such an agent. The identity of this possible activator with the W-substance of Hogben and Slome (1931) or the adrenaline-like material of Abramowtiz (1936) is entirely hypothetical. When a partly blanched catfish such as has just been described is injected with an ordinary ample dose of adrenaline, it regularly blanches to complete paleness showing that its melanophores, though they came to rest at a stage somewhat short of full concentration, are capable of reaching this extreme if appropriately stimulated. The amount of adrenaline in the fish's skin at the semi-pale stage must therefore have been insufficient.

Intermedine, like adrenaline, can excite a full chromatic change, and in this instance from any degree of paleness to full coal-blackness. This agent acts of course in the opposite direction to adrenaline. It has been intimated by some workers that the full coal-black state of the catfish can be induced only by the combined action of the two darkening agents intermedine and acetylcholine, but in my experience intermedine if applied in sufficient strength will of itself fully darken a catfish. Not all catfishes, however, show this reaction. Some stop short of full blackness, nor do they darken further on additional injections of intermedine or acetylcholine (Fig. 1, second fish from the right). Such individuals are incapable of becoming as dark as others, a peculiarity which is resident probably in their melanophores. Notwithstanding these apparent exceptions intermedine of itself appears to be capable of exciting pigment dispersion to a maximum degree in most catfishes.

Acetylcholine, the second darkening neurohumor, is remarkable for a limitation in its activity not seen in either adrenaline or intermedine. When properly protected by eserine acetylcholine can induce darkening in any blanched or partly blanched catfish to about an intermediate stage. In my own tests pale catfishes as already stated were made to darken till their macromelanophores covered an area some 100 microns in diameter. They failed entirely to reach a dispersion of 145 microns, the degree attained in coal-black fishes under the action of intermedine. The turning point for darkening with acetylcholine is a definite one and leaves the fish at a general color stage approximately intermediate (middle specimen in Fig. 1). The states of the macro- and micromelanophores in such a fish are reproduced in Figs. 4 and 5. The macromelanophores under these circumstances are characterized by short, blunt pigment processes and the micromelanophores by short, fine ones.

If into catfishes that have attained this degree of darkening from acetylcholine additional injections of this neurohumor are made, the fishes do not darken further but almost invariably die as they do when strong concentrations of this agent (1 part in 100 or in 1000 parts of solvent) are injected. Thus it appears that acetylcholine will darken catfishes only to an approximately intermediate point and when applied in larger amounts acts as a poison. It is interesting to note that of the three chief chromatic neurohumors in the catfish, acetylcholine is the only one that shows obvious poisonous properties.

It is further the only one that has associated with it a specific destructive agent, cholinesterase, that quickly removes it from the circulation of the fish. This complication may be interpreted from a biological standpoint as a protective measure in the economy of the fish.

When a catfish has been rendered as dark as it can be by acetylcholine an additional injection of intermedine will induce the animal to assume a much darker tint. In fact it may readily be brought by this means to the extreme coal-black stage. For reasons already given this subsequent response must be regarded as nothing more than the typical response to intermedine itself.

The discovery that hypophysectomy is followed by a limitation in the capacity for darkening in the catfish (Abramowitz, 1936; Veil, 1937) and that this limitation is a strikingly definite one (Osborn, 1938; Wykes, 1938; Enami, 1939; Parker, 1939*a*) is an interesting biological phenomenon. Why is it that acetylcholine will carry a melanophore from any condition of concentrated pigment to one of only half-dispersion and intermedine from any such state to full dispersion? The answer to this question is by no means obvious. However the solution is probably to be sought in some relation between the two neurohumors concerned and their direct action on the melanophore.

Immature melanophores in embryonic or larval animals are truly ameboid cells. They creep about within the tissue spaces of the animal that gave rise to them and disperse and concentrate their pigment with striking and irregular change of form. As they mature they lose this protean capacity and in each successive dispersion they reproduce the form of the preceding one. This duplication of outline has been demonstrated by successive photographs of the chromatophores in crustaceans (Perkins, 1928; Brown, 1935). If, however, the photograph of a first dispersed phase is closely compared with that of a second one, as can be done in Perkins' figures (1928, Pl. 1), it will be seen that though the main pigmented branches and sub-branches agree in a remarkable way, the small terminal divisions do not do so. The cause of this terminal diversity is by no means clear. Is it possibly merely an out-of-focus effect in the photograph or may it be an incomplete dispersion of pigment at one phase as compared with the other? I regret to say that I cannot answer this question, particularly in relation to *Ameiurus* where the melanophores are so crowded as to overlap and where therefore the outline of single color-cells cannot be followed as they can be in crustaceans (Fig. 6). After studying Perkins' figures with much care I am of opinion that there is a true diversity among the peripheral and terminal branches of the chromatophore repeatedly expanded as contrasted with the uniformity of its main branches. The larger branches are certainly rigidly constrained, but the smaller, peripheral and terminal twigs appear to retain the plasticity and freedom of movement that characterize whole embryonic color cells. If there should thus prove to be two different regions in the branches of catfish melanophores, one rigid and near the cell body and the other plastic and peripheral, might it not be that such regions are in some way related to the differences in melanophore responses to the two neurohumors concerned? Thus acetylcholine might be limited in its activation to the rigid part of the system whereas intermedine would not only disperse pigment over this portion but activate as well the distal plastic part of the branches. Such a suggestion is of course entirely hypothetical, but it may be helpful in leading to an experimental testing of the remarkable chromatic limitation shown by acetylcholine as contrasted with intermedine.

5. Summary

1. The three chief chromatic neurohumors of the catfish, adrenaline, intermedine, and acetylcholine, all act on the melanophores of this fish directly.

2. Adrenaline, the probable neurohumor of the concentrating chromatic nerves in the catfish, can fully blanch this fish by inducing a complete concentration of its melanophore pigment.

3. Intermedine, a neurohumor from the pituitary gland of the catfish, can fully darken this fish by inducing a complete dispersion of its melanophore pigment.

4. Acetylcholine, the probable neurohumor of the dispersing chromatic nerves in the catfish, can darken a blanched fish to an intermediate dark tint but not further.

5. This limitation in acetylcholine as contrasted with intermedine may depend upon a limitation of its action to what appears to be a basal, rigid part of the melanophore system of branches as contrasted with a peripheral, plastic portion of this system. Both these portions may well be affected by intermedine.

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PLATE 1

All figures are photographic reproductions from the common catfish *Ameiurus nebulosus*.

Fig. 1. Five prepared catfishes showing the range of color change from full pale to coal-black. The coal-black specimen was eyeless; the other four fishes were normal.

Figs. 2 to 7. Photomicrographs of melanophores from preserved catfish tails. All figures are of the same magnification, approximately 110 diameters.

Figs. 2 and 3. Melanophores from the palest fish, extreme left, in Fig. 1. Pigment fully concentrated.

Fig. 2. Macromelanophores, average diameter about 45 microns.

Fig. 3. Micromelanophores, average diameter about 12 microns, with a few macromelanophores out of focus.

Figs. 4 and 5. Melanophores from the intermediate fish, middle one, in Fig. 1.

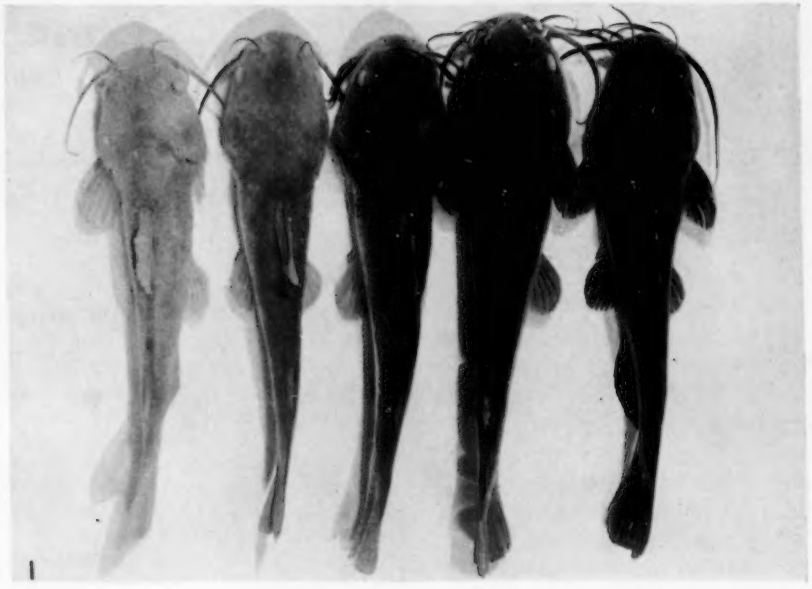
Fig. 4. Macromelanophores, covering areas with an average diameter of about 100 microns.

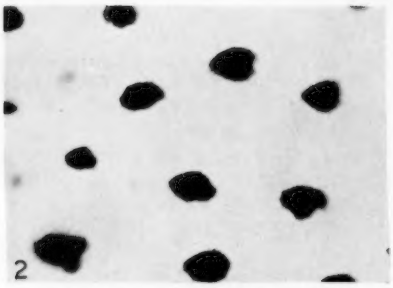
Fig. 5. Micromelanophores; outlines irregular and with scanty, fine processes.

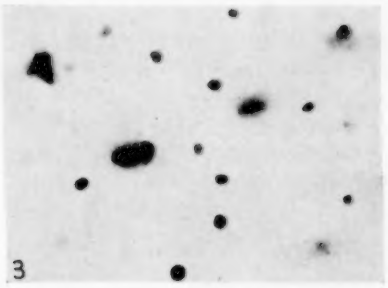
Figs. 6 and 7. Melanophores from the coal-black fish, extreme right, in Fig. 1. Pigment fully dispersed.

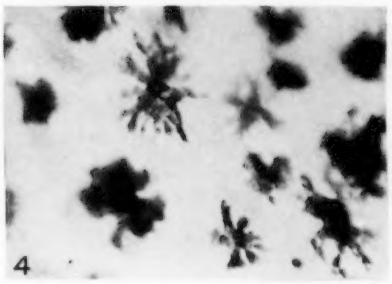
Fig. 6. Macromelanophores, covering areas with an average diameter of about 145 microns.

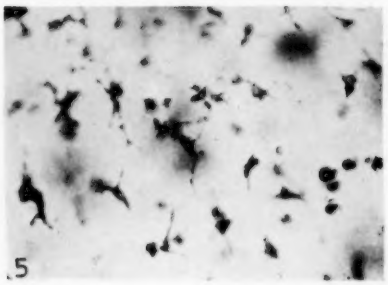
Fig. 7. Micromelanophores, processes of different cells appear here and there to form a network.

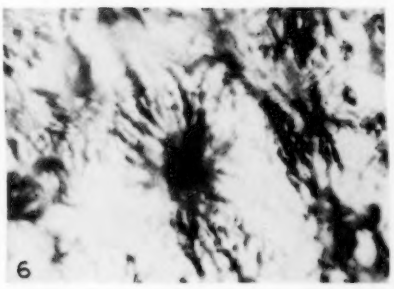












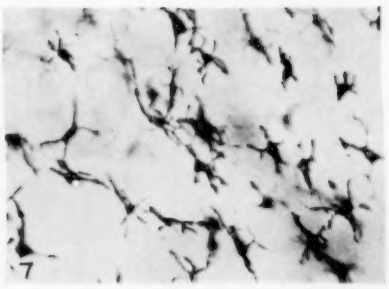


PLATE 1

GENETICS OF PARAMECIUM BURSARIA. II

SELF-DIFFERENTIATION AND SELF-FERTILIZATION OF CLONES[1]

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ABSTRACT

Originally all members of a clone are of the same mating type; hence they do not conjugate together. At very long intervals (about 2,000 culture days), a clone may undergo self-differentiation into two mating types, which then conjugate (self-fertilization of the clone). In any clone self-differentiation yields in all cases the same two mating types, one of which is the original type of the clone. Different clones, even originally of the same mating type, may yield different sets of two types, so that the combination of two types produced by any clone is characteristic for it. In Variety I, with four mating types A, B, C, and D, all the six possible combinations have been observed: AB, AC, AD, BC, BD and CD. The two mating types produced by self-differentiation of a clone may each by renewed self-differentiation produce again the same two types.

The two clones produced from one by self-differentiation differ from each other in (1) mating type; they may also differ (2) in size and form of the individuals; (3) in rate of fission; (4) in readiness to conjugate. The population collected from a given locality often (but not always) consists of just two types, either of which may by self-differentiation produce the other.

By conjugation of the two mating types resulting from clonal self-differentiation, additional mating types are produced. In Variety I, a clone of type D has been observed to produce thus all the four types (A, B, C, D). Clones of type A, B, or C have each produced under observation three of the four types of the variety.

A considerable proportion of the pairs produced by self-fertilization die without reproducing, and this proportion increases as self-fertilization is repeated one or more times.

Through self-differentiation and self-fertilization the sexual reactions of a clone culture may become changed in a number of different ways.

IN the first of these contributions and in other papers by the present author (1938, 1939, 1939a, 1940) it was shown that, in any variety, the individuals or clones of *Paramecium bursaria* belong to a number of different "mating types" which play in the mating behavior the role of diverse sexes. So far as fertilization is concerned the individuals or clones may be looked upon as hermaphroditic: each plays in conjugation the role of both male and female. Each furnishes a migratory pronucleus which passes into the opposite conjugant, corresponding thus to the male germ cell. And each furnishes a stationary pronucleus which receives and unites with the migratory pronucleus, corresponding thus with the female germ cell. And each ex-conjugant continues to reproduce after conjugation has occurred. Fertilization is thus reciprocal. Nevertheless the individuals which conjugate together are physiologically differentiated into diverse mating types which play in the mating behavior the role of diverse sexes. Individuals or clones of the same mating type do not conjugate together; but those of different mating types may and do unite in conjugation.

The species was further shown in previous contributions to include three genetically isolated varieties, members of any one variety not conjugating with members of either of the other two varieties. In Variety I there are four mating types, designated respectively as A, B, C and D. In Variety II there are eight mating types, E, F, G, H, J, K, L, M. In Variety III there are again four, designated N, O, P and Q.

In earlier papers the three varieties have been spoken of as three "groups." As the three appear to be clearly separable, it seems best to use henceforth the designation varieties.

The different types and varieties, it may be remarked, are, in the present state of knowledge, not definable by fixed morphological or physiological characteristics apart from conjugation, but only by the system of inter-conjugation. The types and varieties can be identified with those here designated only by comparing their mating reactions with those of certain cultures now existing in the author's laboratory. Here are preserved living cultures of each of the sixteen mating types that constitute the three varieties. These existing cultures of the different types may be called testers.

To determine the variety to which an unknown clone belongs, its members must be tested suc-

[1] This work was aided by grants from the Carnegie Corporation. To my assistants, Dr. Elizabeth Kirkwood and Mrs. Pauline Opitz, I am indebted for carrying out much of the detailed work.

cessively with testers of at least two of the mating types of each of the three varieties, until some type is found with which the unknown clone conjugates. The latter is thereby shown to belong to the same variety as the tester with which it conjugates. To determine next the mating type to which the unknown clone belongs, its members must be tested successively with testers of each of the mating types of that variety. If for example the unknown clone belongs to Variety I, some of its members must be mingled (separately) with each of the four testers of mating types A, B, C and D. Invariably (if it is mature) it is found to conjugate with three of the types but not with the fourth. This type with which it does not conjugate is the mating type to which it belongs (since individuals of the same type do not conjugate together). If the unknown clone is immature, the variety and type to which it belongs cannot be determined until it has become mature.

Except at certain rare periods, to be described, all members of a clone (derived by fission from a single parent individual) belong to the same mating type, so that members of the same clone do not conjugate together. The mating type is thus in ordinary fission inherited without change. For these relations, therefore, *the clone is the unit of observation and discussion.* All its individual members belong to the same mating type, so that one clone of Group I is of the type A, other clones are of the type B, others of C or D. For brevity, therefore, two clones will be spoken of as conjugating together; this means of course that the individual members of the two clones conjugate when they come in contact.

Self-Fertilization of Clones

Although members of the same clone do not ordinarily conjugate together, very rarely conjugation is found to occur among members of a single clone, so that the clone is self-fertilized. This self-fertilization of clones forms the subject of the present contribution. It has important consequences for the genetics and general biology of the organisms, and the processes accompanying it throw much light on the genetic constitution of the animals.

Frequency of Self-Fertilization; Proportion of the Stock that Undergoes It

Self-fertilization of clones is evidenced by the appearance of conjugating pairs in cultures consisting of but a single clone. In *Paramecium bursaria* it occurs only at rare and seemingly irregular intervals. Usually when it occurs but a few pairs are to be seen among great numbers of nonconjugants. Many clone cultures may be kept under observation for long periods—for months or years—without any indication of self-fertilization. At unexpected times however pairs may be found in a clone culture, showing the occurrence of self-fertilization in the clone. Data are herewith presented to indicate the frequency (or infrequency) of its occurrence:

(1) From the population *He* (Variety I), collected April 21, 1938, twelve clones were cultivated, from twelve isolated individuals. These were kept under daily observation for more than a month. During the period of observation self-fertilization occurred in but one of the twelve (clone He6).

(2) From the population *Br*, 103 clones of Variety I were isolated November 18, 1939. These were kept under daily observation throughout the rest of November, all of December, and a part of January, 1940, somewhat more than six weeks. During this period self-fertilization occurred in but one of them—the clone Br48.

(3) From the population *DM* sixty clones were isolated December 24, 1939. These were kept under daily observation for one month. During this period, a single pair was found in the clone DM13, six pairs in the clone DM42. In the other 58 clones self-fertilization did not occur.

(4) From the population *San*, seventy-two clones were isolated December 24, 1939: of these, 71 belonged to Variety II, while one belonged to Variety I. The cultures of these clones were kept under daily observation from January 9 to February 14, 1940, a period of 36 days. Self-fertilization did not occur in any of them during this period.

(5) On August 2, 1940, 38 clones of Variety II were placed each in a Syracuse dish culture. These 38 clone cultures were kept under daily observation until October 20, a period of 79 days. During this period self-fertilization occurred in but one of the clones (clone S, of mating type J).

(6) From November 9, 1940, to January 14, 1941, a large number of cultures were kept under observation, examinations being made either every day or every alternate day. These were cultures in Syracuse dishes; they included 178 cultures of clones of Variety I, 224 of Variety II, and 63 of Variety III. Thus a total of 465

cultures were kept under observation for a period of 66 days. Among these certain clones were represented by several cultures, most of them by but one culture.

During the period of 66 days self-fertilization was detected by the presence of pairs in 15 of the 465 cultures. These 15 included 9 different clones.

From this last case some approximate idea may be obtained of the frequency of self-fertilization. There were 465 cultures observed throughout 66 days, making a total of 30,690 culture-days. During this time 15 of the cultures showed self-fertilization, or at the rate of one self-fertilization for 2,096 culture-days. This would be a rate of one self-fertilization per culture in 5.7 years.

When self-fertilization is detected by the presence of pairs in a clone culture, the proportional number of individuals undergoing self-fertilization varies greatly in different cases. In many cases but a single pair, or two or three pairs, are to be found in a culture containing many thousand individuals. From such a minimum the proportional number varies in different cultures up to cases in which a large majority of the individuals are undergoing clotting and pairing.

Furthermore, at times there occur epidemics of self-fertilization which continue in a given culture for several days or even weeks, with intervening periods in which no pairs are to be found. In such continuing epidemics the number of pairs to be found at any given time varies in different cultures from one or a few to very great numbers. The following specific cases will illustrate the varied conditions found:

(1) In a populous culture of the clone DM22 (Variety I) a single pair was found January 10, 1941. This clone had been under close observation from November 9, a period of two months, and was examined with care daily for 25 days after January 10, but no more pairs were found in it.

(2) A culture of the clone GM140*a*1 (Variety II) was kept under observation from November 9, 1940. No pairs were found until January 14, when a single pair was found and removed. Daily examinations showed no more pairs until January 21, when 13 pairs were found and removed. On the next day another pair was found. No more occurred, though daily examination was continued till February 3.

(3) The clone LP10 (Variety I) was represented by 6 cultures in Syracuse dishes. These were kept under observation from November 7, 1940, without the occurrence of pairs, till January 3, 1941, when six pairs were taken from culture 1. No pairs were present thereafter till January 6, when 2 pairs were found in the same culture, and removed. There were no more pairs till January 19, when 16 pairs were observed (and removed) in culture 1. On January 20 there were 3 pairs (removed); on January 22, 5 pairs (removed). All these were from culture 1: the other 5 cultures contained no pairs at any time. All were kept under daily observation until February 4, but no more pairs were found in cultures of this clone.

(4) In a culture of the clone 2H51*a* (Variety I) several pairs were observed and removed on each of the following days: January 5–6–8, 1940. On January 19, 20, 21 and 22 very numerous clots and pairs were present, so that conditions in the culture resembled those in mixtures of two populous clones of different type. Occasional pairs continued to be found until January 27; at this time the epidemic of conjugation was over.

No method has been discovered for producing self-conjugation of a clone at will. Apparently self-fertilization is to some extent favored by the conditions resulting from the following procedure. The clone is recultured by removing large numbers of its individuals to a new culture medium. Four or five days later the conditions seem favorable for self-fertilization, in case the clone is ripe for that operation. If some twenty-five clones are thus treated on a given day, it is possible that one or two of these will yield pairs in the course of the next four or five days. But even such a proportion is by no means always successful.

Self-Differentiation Preceding Self-Fertilization

All the members of a clone usually belong to the same mating type. When self-fertilization occurs, do the two individuals that unite in conjugation still belong to the same type, so that conjugation occurs between individuals of a single type? Or does there first occur a differentiation of the clone into two different types which then conjugate, as occurs in *Paramecium aurelia* (Kimball 1939, Sonneborn, 1939)? In the species just named there are but two mating types in any variety, while in *Parmecium bursaria*

there are four or eight, so that there are several different possibilities as to differentiation.

The answer to these questions is discovered by isolating the two members of "split pairs" and testing them. That is, before conjugation has been consummated the two individuals that were beginning conjugation are separated and isolated; then by appropriate tests the type of each is determined. This was practiced extensively in self-fertilizing clones of *Paramecium bursaria*. It reveals many important biological relations.

The delicate operation of isolating two individuals that are in the preliminary stages of conjugation is facilitated by special features of the process of clotting or agglutination which constitutes the first step in conjugation (see Jennings 1939, 1939*a*, for descriptions and figures of this clotting). At first two individuals merely adhere together slightly by any parts of the body surface that happen to come in contact. Often the anterior or posterior tip of one individual adheres to a tip or side of another, so that when one of the individuals moves, the other is pulled along with it. Before conjugation is finally consummated the two individuals must become placed side by side with oral surfaces in contact, a process requiring time (often an hour or more). But the early slight adhesion occurs only between individuals that can conjugate together, individuals that normally would conjugate together.

Two individuals thus lightly adhering in the early stages of conjugation are taken up with capillary pipette and removed to an isolated drop of fluid. In the process the two always become separated; they are then placed apart in different drops. The mating type to which each belongs is then determined by tests with individuals that are of known mating type.

The tests for determining the mating type of the two members of the split pair may be applied directly to the two individuals that were beginning conjugation. Or these two individuals may be cultured, each producing a numerous clone; then the tests may be applied to these clones. This latter method is much more convenient and gives the same results as the other. The general method of testing has already been described. Each member of the split pair must be tested with all the types of the variety to which it belongs. Thus, to determine the types to which the two members of a split pair belong, eight tests are required in case of Variety I, or Variety III, sixteen in case of Variety II.

Unfortunately for the convenience of study, self-fertilization occurs very rarely, and no means have been discovered for inducing it or hastening its occurrence. To investigate it therefore one is compelled to take advantage of such opportunities as present themselves spontaneously while one is cultivating and observing large numbers of cultures. Some hundreds of clone cultures were in progress at all times in our laboratory. The observations and experiments on self-fertilization herein set forth were made possible by this fact.

Self-Differentiation and Self-Fertilization in Variety I.—Self-fertilization was first examined by the aid of split pairs in a clone known as He6. Twelve clones were derived from the multiplication of 12 isolated individuals that were collected April 21, 1938; these twelve were designated He1 to He12. They were found by tests to belong to Variety I. On May 7, 1938, self-fertilization was found to be occurring in the clone He6, as shown by the presence of pairs. This clone was of the mating type D. Self-fertilization did not occur in the other eleven clones of He during the period of about a month during which they were examined daily.

To determine whether the clone He6 had before self-fertilization differentiated into different mating types, 18 split pairs were obtained in the way described above. The two members of each split pair are designated as *a* and *b* respectively—so that in each case *a* and *b* had begun to conjugate together. From each of the 36 members of the 18 split pairs (18*a* and 18*b*), a clone was obtained by allowing the individuals to multiply. The mating type of each clone was then determined by testing. In addition, the mating type of the two original individuals (*a* and *b*) of three of the pairs was determined directly.

The direct determination of the mating type for the single individuals *a* and *b* that have begun to conjugate is a delicate operation. For its successful performance, the individuals of the clone from which the split pairs are taken must differ in color from the individuals of the testers, so as to be distinguishable from the latter. This is not unusual; the clone from which the split pairs are taken may be much lighter green in color, in consequence of recent rapid multiplication, or in consequence of having been kept for some days in the dark. The required determination is made as follows. The individual *a* of the split pair is placed in a dense group of individuals of the type A. The individuals of type A either

adhere to the individual *a* or they do not. As soon as this is discovered, and before there is actual union between *a* and any individual of type A, the individual *a* is removed with fine pipette and tested in the same way in a dense group of individuals of type B. Next it is tested similarly with C, then with D. Three of the types attempt to unite with the individual *a*, while one does not; this latter is the type to which *a* belongs. A similar series of tests is made with the individual *b* of each pair.

In each of the three split pairs thus tested, one of the two members was found to be of the original type D, while the other was now of type A.

The 36 clones derived from *a* and *b* of the 18 split pairs were tested May 9 and 10, after each member (*a* and *b*) had produced a clone of many individuals. The tests were made as usual by mingling a part of the clone *a* with testers of A, other parts with testers of B, C and D. Tests with clone *b* of each split pair were made in the same way.

In every one of the 18 split pairs it was found by this method that the clone derived from one member of the pair was of the original mating type D, while the clone derived from the other member was of the mating type A. The six clones of the three pairs that were previously directly tested gave the same results as in the direct tests. That is, the clone derived from the single individual (*a* or *b*) of the split pair remains of the same type as its single ancestor. The mating type is inherited in vegetative descent from members of split pairs, just as in the usual vegetative reproduction.

The clone He6, therefore, originally of the matting type D, has before self-fertilization differentiated, some of its members transforming into type A. The two types D (original) and A (derived) then conjugate together.

Mating is thus between individuals of different mating type, even though all the individuals belong originally to the same clone (in this case the clone He6). Self-fertilization is preceded by self-differentiation into two types, an original type (D) and a derived type (A).

Self-Differentiation and Self-Fertilization in Variety II.—Somewhat more than a year later this matter was further examined in a clone of Variety II. In this variety there are eight mating types, as compared with four in Variety I.

Four clones were derived from four individuals collected in the Botanical Garden of the Johns Hopkins University, August 20, 1939. These four were designated JH1, 2, 8 and 13. Within a week, and before they had been tested to determine the group and types to which they belonged, many pairs were found to be present in the clone culture of JH2, so that self-fertilization was occurring (August 27, 1939). The four clones were carried to California, and tests made September 14, 1939, showed that all belonged to Variety II. The clones of JH8 and JH13 were found to be of type E, the clone JH1 of type F. The clone of JH2, having undergone partial self-fertilization, no longer gave clear results as to the type to which it originally belonged.

On September 27, 1939, there were again pairs in clone JH2, so that it was again undergoing self-fertilization. Eighty-five split pairs were obtained and their two members isolated on that date. Clones were obtained by cultivating each of these, record being kept of each separately and of the pair to which it belonged. The clones of the different pairs were labeled S1*a*, S1*b*, S2*a*, S2*b*, and so on, up to S85*a* and S85*b*. The 170 clones were then tested with each of the 8 types of Variety II. In each of the 85 pairs one clone was of the type E, the other of type F. The clone JH2 had differentiated into these two types, which then conjugated. In this case it is not known to which of the two types the clone belonged at first.

On October 6, 1939, the clone derived from one member of the split pair S4 (above) was found to be undergoing self-fertilization. This clone, S4*a*, was of the mating type E. Six split pairs were obtained from it; and from the two members *a* and *b* of each, clones were cultivated. Tests showed that in each case one member of the pair was E, the other F. In addition, while this clone S4*a* was thus differentiating into two types, fifteen single free individuals were isolated from it (October 7, 1939), and from these, fifteen clones were obtained. Tests made October 16 and 17 showed that ten of these were of type E, while 5 were of type F.

In this case, therefore, the clone S4*a*, originally of type E, has partly transformed into type F; then in many cases the two have conjugated. Also single free individuals of each of the two types are present (October 7).

One of the clones of type F, derived from one of the single free individuals above mentioned, underwent self-fertilization October 16 and 17. From it 24 split pairs were obtained and clones were cultivated from the two members (*a* and *b*)

of each. In every one of the 24 one member was found by test to be of F, the other of E. In this case a clone that was originally of type F has at self-differentiation transformed partly into type E.

Thus clones that are originally E differentiate into E and F, as do also clones that were originally F. In each case the clone is seemingly of the constitution (EF); such a clone (EF) may manifest itself either as E, or, after transformation, as F. In the case described there has been a double transformation: a clone of type E (clone S4a) differentiated into E and F; then one of these F clones (produced from E) again differentiated into E and F. This double transformation is of much significance for the interpretation of the differentiation—a matter to be dealt with later.

In all the cases described above, it will be observed that every split pair from any clone consists of the same two types. The 18 split pairs from clone He6 each consisted of D and A. Of the 115 split pairs derived finally from the clone JH2 each consisted of E and F. Thus any single split pair shows the two types into which any clone differentiates. This situation was found in all the cases in which self-fertilization occurred in a clone known not to have become differentiated beforehand, in consequence of an earlier self-fertilization (for the results of successive self-fertilizations see a later section).

Thus, to determine the type into which an existing uniform clone differentiates, large numbers of split pairs are not required. In some later studies, therefore, smaller numbers were employed, though usually six to twenty split pairs were examined.

In the course of the years 1938–1941, self fertilization was observed and analyzed by the use of split pairs in 32 clones, of which 15 were of Variety I, 17 of Variety II. Self-fertilization was observed also in two clones of Variety III, but these were not studied by means of split pairs.

In tables 1 and 2 the chief data on the self-fertilizing clones will be found summarized for varieties I and II respectively. The tables include at the left a list of the clones, next the dates

TABLE 1

SELF-DIFFERENTIATION AND SELF-FERTILIZATION OF CLONES IN *Paramecium bursaria*, VARIETY I

List of clones in which self-differentiation and self-fertilization were observed, with dates, number of split pairs examined, the original mating type (if known), and the two mating types resulting from differentiation.

Designation of Clone	Dates	Number of Split Pairs	Original Type	Mating Types Resulting
He6	May 4, '38	18	D	D and A
J17*b*1	Nov. 7–12, '39	60	B	B and D
2H58*a*	Nov. 11, '39	33		B and A
Br48	Nov. 29–Dec. 4, '39	13	B	B and C
2H34*b*	Dec. 1–14, '39	13		B and D
2H54*b*	Dec. 1–14, '39	22		B and D
2H51*a*	Jan. 5–11, '40	8	B	B and C
2H51*a*	Jan. 19–20, '40	18	B	B and C
2H51*a*	Jan. 21, '40	14	B	B and C
DM13	Jan. 31, '40	1	D	D and C
Lo1	Nov. 7, '40	6	D	D and C
Lo1	Nov. 12–15, '40	7	D	D and C
LP10	Jan. 3, '41	4	D	D and C
Wat 1–S6*b*	Jan. 13, '41	9	B	B and C
Wat 1–S13*a*	Jan. 17, '41	12		A and B
Lo12	Feb. 2, '41	19	C	C and B
Lo12–S21*a*	Feb. 27, '41	11	C	C and B
BD214	Mar. 4, 5, '41	11		A and D

Note: In the clones 2H58*a*, 2H34*b* and 2H54*b* the original type was practically certainly B. But tests were not made till after self-differentiation had occurred. In all cases, however, (1) the other ex-conjugant of the pair (2H58*b*. 2H34*a*, 2H54*a*) was B, and it is known that in 98 per cent of all cases the two ex-conjugants of a pair are the same in mating type; (2) after self-fertilization had begun a very large majority of the individuals of the clone were in each case of type B: these were presumably the individuals that had not differentiated. In clone BD214 tests had shown that the original type was not D. It must therefore have been A, though it is not so listed in the table because the proof that it was A is only indirect.

TABLE 2

SELF-DIFFERENTIATION AND SELF-FERTILIZATION OF CLONES IN *Paramecium bursaria*, VARIETY II

List of clones in which self-differentiation and self-fertilization were observed, with dates, number of split pairs examined, the original mating type (if known), and the two mating types resulting from differentiation.

Designation of Clone	Dates	Number of Split Pairs	Original Type	Mating Types Resulting
JH2	Sept. 27, '39	87		E and F
JH2–S4*a*	Oct. 7, '39	6	E	E and F
JH2–S4*a*1	Oct. 16–17, '39	24	F	F and E
Mo1	Nov. 28–Dec. 5, '39	16	F	F and M
Mo3	Nov. 28–Dec. 5, '39	10	M	M and F
Mo4	Dec. 4–7, '39	7	M	M and F
Mo2	Dec. 4–7, '39	3	F	F and M
NH13	June 27–July 6, '40	See note	H	H and E
S	July 6, '40	10	J	J and L
S	Aug. 15–16, '40	5	J	J and L
GM215*a*2	July 6, '40	3	K	K and F
Fd	Dec. 12–27	21	E	E and F
HV3	Dec. 2–Jan. 6, '40	15	F	F and G
HV3–S4*b*	Jan. 6, '40	10	G	G and F
F1	Dec. 27, '40	1	G	G and F
GM140*a*1	Jan. 22, '40	1	H	H and K
Coq5	Feb. 27–March 1, '41	12	F	F and G
Coq5–S9*a*	March 12–13, '41	7		F and G

Note: In clone NH13, the types resulting from self-differentiation were determined, not from split pairs, but from the two types of individuals present in the culture while self-fertilization was occurring.

at which self-fertilization occurred, the number of split pairs tested in the manner described above, the mating type to which the clone belonged before self-differentiation, and in the last column the two mating types which resulted from self-differentiation (the original and the derived types).

Before taking up the general relations involved, certain special features of the tests may be mentioned.

At times, instead of a pair of individuals beginning conjugation (a "split pair"), a small clot of three or four individuals clinging together is taken from the self-fertilizing culture. Each of the three or four individuals is isolated, a clone obtained from it, and this is tested in the usual way. Invariably such a small clot contains individuals of just the two mating types that compose any split pair. Thus in the case of the self-fertilizing clone 2H51*a* of Table I the split pairs all consisted of one individual of type B and another of Type C. In one case there was a clot of 3; in this two individuals were of type B, one of type C. Again, the clone S (Table 2) yielded at self-fertilization split pairs that consisted of the two types J and L. In four cases clots of three were investigated; in each case two of the individuals were of type J, one of type L. Such relations were found in all the many cases of this kind that were examined.

A possible source of error in isolating two individuals that are in the preliminary stages of conjugation lies in the fact, described in earlier papers (1939, 1940) that often there is close and continued contact between two individuals that do not finally conjugate. In such association the two individuals in contact swim together in a coordinated way, but there is no physical cohesion between the two as there is between two individuals that will finally conjugate. With practice this enables the operator to distinguish between the two cases. In the preliminary stages of conjugation there is actual adhesion, so that the motion of the two individuals is often not coordinated. One pulls the other out of its normal course in consequence of the irregular adhesion. Only pairs in which this occurs are certainly in the preliminary stages of conjugation. Unless care is exercised, however, individuals that are merely swimming together in contact may be taken up in place of pairs that are actually adhering. This is more likely to occur in the common case that the culture contains very few adhering pairs, so that the investigator is anxious not to miss any of the possible cases. If two individuals, not adhering,

but merely in contact, are taken up and isolated, it is found upon test that they both belong to the same mating type; we are then not dealing with a genuine "split pair". In practice such cases are rare; they have occurred but a few times among the hundreds of split pairs that have been examined.

Very old clones in which self-fertilization has occurred long ago, so that the descendants of the pairs formed have become mature, give results which are diverse from those presented in Tables 1 and 2. Such cases are described later.

In Table 1, of Variety I, it may be observed that in none of the clones listed was the original mating type certainly A, and from this the inference might be drawn that type A does not undergo self-differentiation and self-fertilization. However, self-fertilization had been observed in two clones of mating type A (namely clones *l* and 88*a*1) before the method of analysis by means of split pairs had been devised. Also it is practically certain, on indirect evidence, that in clone BD214 of Table 1 the original type was A.

General Relations in Self-Differentiation

We shall take up the general relations in self-differentiation as illustrated by the cases summarized in Tables 1 and 2, giving such additional facts as are pertinent.

1. In all the cases analyzed by the use of split pairs, it was found that self-differentiation of the clone had occurred before self-fertilization. All members of the clone are originally of a single mating type. At a certain period part of its members transform into a second or derived mating type; then individuals of the two types agglutinate and conjugate. Conjugation is thus, in all cases observed, between individuals of different mating type.

What is the nature of the process by which self-differentiation into two types occurs? It seems probable that some nuclear change underlies the differentiation. In *Paramecium aurelia* such a change of type is produced at endomixis. In *Paramecium bursaria* endomixis occurs very infrequently (Erdmann 1927) and seemingly not at regular intervals. This is likewise the case with self-differentiation and self-fertilization. One is led to suspect, therefore, that endomixis or some similar nuclear change occurs at the time of self-differentiation. A cytological investigation is obviously needed. The phenomena present great difficulty, since self-differentiation is very infrequent, and is not outwardly manifested. Moreover, it is possible that there is a period of immaturity following self-differentiation, so that when self-fertilization is discovered through the presence of pairs in the clone culture, the nuclear processes of self-fertilization may have been long completed. The conjugation which follows self-differentiation of course includes complex nuclear changes which would make detection of the nuclear changes peculiar to self-differentiation very difficult, in case the two are occurring at the same time in the clone culture.

2. An important feature of self-differentiation appears in Tables 1 and 2. In any culture of a particular clone, self-differentiation yields in all cases the same two types (rare apparent exceptions appear in old clones in which self-differentiation has occurred long before the split pairs are obtained, but such are not real exceptions, as explained later).

In *Paramecium bursaria*, as before mentioned, there are more than two mating types. In Variety I there are four, in Variety II there are eight. A particular two out of these four or eight result from the self-differentiation of any clone culture. In clone He6 the two types produced are always D and A, never either types B or C. The original type of this clone is D: self-differentiation adds to it the derived type A. In the clone of JH2 of Variety II self-differentiation yields in all cases the two types E and F, never any of the other six types (G to M). A culture that is first of type E produces only the additional derived type F, while a culture that is at first of type F produces only the additional derived type E. Similar relations hold for all the cases of self-differentiation analyzed and summarized in Tables 1 and 2.

3. Do all clones of a certain original type, as D, produce at self-differentiation a particular derived type, as A (as in clone He6 above)? In other words, are two particular types always associated together in the constitution of a clone, as D and A are in the constitution of the clone He6, or as E and F are in the constitution of the clone JH2?

These questions are answered in the negative by the data of Tables 1 and 2. Table 1 contains several cases of self-differentiation in clones that were originally of mating type D. The first of these listed (He6) gave rise at differentiation to the derived type A, while several others produced type C. In some cases in which the original type

is uncertain, the two types D and B are associated as the products of differentiation. Again self-differentiation was observed in six clones that were originally of type B. Of these five produced the derived type C, while one produced the derived type D. In another case (clone 2H58*a*) B and A were the products of differentiation.

In Variety II (Table 2) self-differentiation was observed in five clones that were originally the mating type F. Two of these produced at self-differentiation the derived type M, one produced type E and two produced type G. Two clones were originally H; one differentiated into H and E, the other into H and K.

It is clear, therefore, that different clones originally of the same mating type may and do at self-differentiation give origin to different derived types.

4. Thus any particular clone is so constituted as to yield at self-differentiation a particular combination of two mating types, different clones being diverse in the combinations which they produce. The combination produced by any clone is characteristic for that clone. The constitution of the clone may be designated by the pair of types that it produces. The clone He6 was of the constitution (DA): it could manifest itself either as mating type D or as A. Using this notation, we have in Table 1 and 2 clones of the following constitutions:

Variety I: (AB), (AC), (AD), (BC), (BD), (CD).
Variety II: (EF), (EH), (FG), (FK), (FL), (FM), (HK), (JL).

Thus in Variety I all the six possible combinations of two types have been found to occur. In Variety II, of the 28 possible combinations of two types from eight, eight have thus far been found. It seems probable that in Variety II, as in Variety I, all the possible combinations of two types may ultimately be found to occur.

5. When a given clone undergoes self-differentiation at successive periods or when in a single clone self-differentiation occurs in two or more different cultures, in each case the same two types are produced.

Thus the clone JH2 underwent self-differentiation at three successive periods: September 27, October 6 and October 17, 1939. At each of these periods, it gave rise to the same pair of types, E and F. Similarly the clone S underwent self-differentiation July 6 and again August 15, 1940. It produced in both cases the same two types J and L. The clone 2H51*a* differentiated January 6 to 8 and January 19 to 21, 1940. In both periods it gave rise to the two types B and C.

In some observed cases self-fertilization in a particular clone occurred in two different cultures which had been cultivated separately for a considerable time. Such are the following:

Clone S, of type J, Variety II. This clone existed in a number of cultures. On July 6, 1940, pairs were present in one of these; on August 15 to 16, pairs were present in another. The two cultures had been cultivated separately for more than six weeks, probably for much more. Split pairs from both consisted of the same two types, J and L.

Clone F*d*, of type E, Variety II. December 12–14, 1940, there were pairs in two cultures which had been cultivated separately since May 4, 1940; that is, for 7 months and 8 days. Split pairs showed that the two differentiated into the same pair of types, namely E and F.

Thus a given clone retains its constitution (as to mating type) certainly for long periods, so that when it differentiates it produces throughout the same two types. Clone JH2 yields in all observed cases types E and F; clone F*d* also yields E and F. Clone S yields J and L, clone 2H51*a* yields B and C, and so on.

6. After a clone has by self-differentiation produced two mating types, each of these may later by renewed self-differentiation again produce the same two mating types. This is exemplified in clones JH2 and HV3 (Variety II) in Table 2. The clone JH2 produced at self-differentiation the two mating types E and F. Clones of each of these types so derived later again differentiated, each producing anew the types E and F. The clone HV3 was originally of the mating type F. At self-differentiation it produced the derived type G, and a clone of this type (designated HV3–S4*b*) by renewed differentiation produced the original type F. Thus after self-differentiation of a clone, each of the two types produced may still produce the other.

The fact that a clone of given mating type may by self-differentiation produce a particular additional mating type presumably rests upon the fact that these organisms are diploid in their chromosomal constitution. After self-differentiation the two clones produced must be still diploid, since each can still produce two different

mating types. This is consistent with the cytological processes that occur in endomixis. Endomixis includes a division of the micronucleus, presumably reductional. Then, according to the recent work of Diller (1936) there is a union of two of these reduced nuclei, to form anew a diploid nucleus ("autogamy"). The occurrence of autogamy at endomixis is confirmed genetically by the observations of Sonneborn (1939) in Paramecium aurelia. The two types resulting from endomixis are consequently diploid, as was the parent clone before self-differentiation.

Self-differentiation might conceivably occur in two different ways. (1) On the one hand a single individual might through endomixis produce both the types that result from self-differentiation. The two types would then presumably separate at a fission occurring immediately after endomixis; this is what occurs in Paramecium aurelia (Sonneborn 1937). (2) Or single individuals of the original type might transform at endomixis into the derived type, leaving other individuals of the original type unaltered. In this second case the "original" and "derived" types would differ in the time that had elapsed since a differentiation had occurred; it might then be expected that the original type would differentiate anew earlier than would the derived type.

This appears to be actually the case in Paramecium bursaria. After self-differentiation has occurred, the clone derived from the original type (obtained from split pairs) is very frequently observed to undergo again self-differentiation, while the clones from the derived type very rarely do so. Among all the cases recorded in Tables 1 and 2, renewed self-differentiation in the clone of derived type was observed but twice, in the clones of JH2 and HV3, of Variety II. Renewed self-differentiation in the clones of the original types was on the other hand observed many times.

7. After self-differentiation of clones into morphologically and physiologically diverse types, the two types into which a clone differentiates are of course physiologically different, in the very fact that they are diverse mating types. The clone He6, originally of type D, produces the derived type A, which reacts in conjugation in a different way from D.

Do other differences, physiological or morphological accompany the physiological diversity of mating type? Evidence on this point gradually accumulated, as follows:

Differences in Size and Form.—It was frequently noticed that in the pairs formed in the process of self-fertilization, one of the two united individuals is larger than the other. This was at first thought to be a transitory phenomenon, without further significance. But some attention was given to it, at first in a rather loose way; later when it was found to be significant it was examined with care.

On November 11, 1939, the clone 2H58*a*, almost certainly of type B, underwent self-fertilization. Thirty-three split pairs were obtained. In most cases one of the adhering individuals appeared to be smaller than the other. Where this was true the larger member of the split pair was in a routine way placed in the left-hand depression of the slide and designated *a*, the smaller being transferred to the right-hand depression and designated *b*. From each of the 66 individuals of the 33 split pairs (33*a* and 33*b*) a clone was obtained, and when the 66 clones had sufficiently multiplied, each was tested to determine the mating type. Of the 33 *a*'s (larger), 30 were of the original mating type B; of the 33 *b*'s (smaller), 30 were of the derived type A. In only three pairs were the relations reversed; *a* was of the derived type A, while *b* was of the original type B. In these three exceptional cases presumably the difference in size (if any) was so slight that they were not correctly classified.

Thus in this clone at the time of conjugation the individuals of the original type B were larger (in the great majority of cases, at least) than those of the derived type A. Not only this, but in the clones derived by fission from the split pairs it was noticed, to my surprise, that the differences in size persisted; they were inherited in vegetative reproduction.

This led to a more exact study of the matter in some later cases of self-fertilization. The clone 2H51*a*, of mating type B, underwent self-fertilization during a considerable period beginning January 5, 1940. From this clone 40 split pairs were obtained. In each pair one member was of the original type B, the other of the derived type C.

Eighteen of these split pairs were obtained January 20, 1940. Opportunity was then taken to make a more exact study of differences in size and form, and their significance. It was observed that in all of the 18 pairs one member was slightly longer and more slender, the other shorter and relatively thicker. The long slender

member was in each case designated *a*, the short thick one *b*. From each of the 36 individuals a clone was derived, and the members of the clones showed the same morphological characteristics as the individuals from which they were derived. The members of all the eighteen *a* clones were long and slender; the members of the eighteen *b* clones were short and thicker. Later the thirty-six clones were tested for determination of the mating type. In every case the *a* clone, of long slender individuals, was of the original type B; while the *b* clone, of shorter thicker individuals, was of the derived type C.

Eight other split pairs from the same source had been obtained earlier (January 6 to 8) and clones derived from them were in existence, but had not been tested. It was decided to try whether it would be possible by means of the differences in size and form to classify these correctly into the two mating types B and C. In each of the eight the clone from one member of the pair consisted of individuals that were long and slender, and therefore presumably of type B. The clone from the other member consisted of shorter thicker individuals; these clones were presumably of the derived type C.

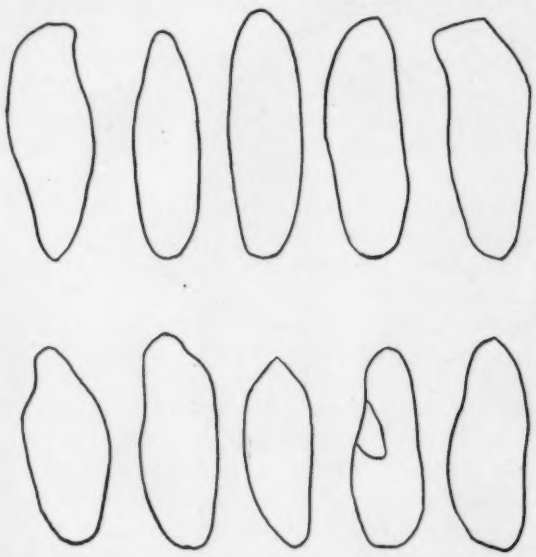

FIG. 1. *Paramecium bursaria*. Camera outlines of typical specimens resulting from self-differentiation of the clone 2H51*a*; all drawn to the same scale. The upper row shows typical individuals of the original mating type B (larger, more slender); the lower row typical individuals of the derived mating type C (smaller and proportionately thicker). From two clones derived from the two members of a split pair isolated January 8, 1940 and cultivated separately till February 6, 1941, on which date they were fixed and drawn. Magnification, 288 diameters.

Later tests confirmed these attributions. In the two sets together there were 52 clones descended from the two differing members of the 26 pairs. In every pair the clone of long slender individuals was of the original type B, while the clone of shorter thicker individuals was of the derived type C.

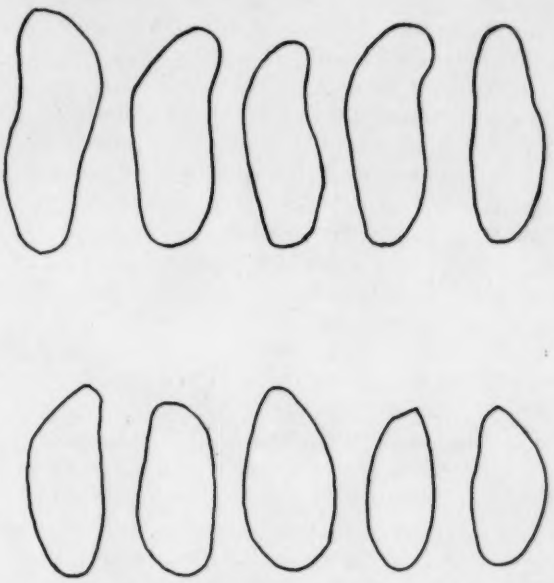

FIG. 2. *Paramecium bursaria*. Camera outlines of typical specimens resulting from self-differentiation of the clone 2H54*b*; all drawn to the same scale. The upper row shows typical individuals of the original mating type B (larger, more slender), the lower row typical individuals of the derived mating type D (smaller and thicker). From two clones derived from two members of split pairs isolated December 10, 1939, and cultivated separately till February 7, 1941, on which date they were fixed and drawn. Magnification, 288 diameters.

These differences (in possibly slightly less marked form) continued to be characteristic for the clones of the two different types for more than a year. Fig. 1 shows in juxtaposition camera outlines (at the same magnification) of characteristic groups of individuals from B and C clones derived from a split pair (Number 9) of this stock; as they were on February 6, 1941; more than a year after the clones were isolated.

Split pairs obtained from the clone 2H54*b* (December 1 to 14, 1939) showed the same sort of differences between the two members. In this case the original type was probably B, the derived type D. The individuals of the clones of the original type B were long and slender; those of clones of the derived type D were short and thick. These differences still persist more than

thirteen months after the clones were originated from the two members of split pairs. This is shown in Fig. 2. Here are camera outlines, at the same magnification, of characteristic individuals of the two types, on February 7, 1941.

Fig. 3 shows further a set of pairs from this clone 2H54*b*, showing the difference in size. In each case the larger member is of the original type B, the smaller member of the derived type D. These pairs were obtained February 7, 1941, by mixing members of clones from split pairs obtained December 10, 1939.

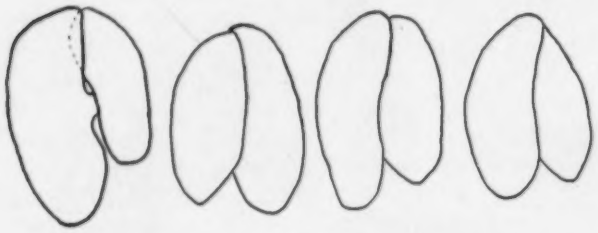

FIG. 3. Camera outlines of a number of pairs from self-fertilization in clone 2H54*b*, showing the difference in size of the two members. In each pair the larger member is of the original mating type B, the smaller one of the derived mating type D. Pairs obtained February 7, 1941, by placing together clones derived from two members of split pairs isolated December 12, 1939 and cultivated separately till February 6, 1941. Magnification, 288 diameters.

In the above described clones of Variety I, therefore, self-differentiation of the clone produces two types that differ not only physiologically, as to mating type, but morphologically, as to size and form. Those that remain of the original type (B in the cases above described) are longer and more slender than those that have been transformed into the derived type (A, C or D in the three different clones described). These differences are perpetuated in vegetative reproduction, so that they still persist in the clones derived from the individuals of the split pairs.

Variety II was less studied from this point of view. But in a clone S of this variety there were indications of a difference in size between the two types produced at self-differentiation. In this case, however, it was the derived type which had the larger individuals. The clone S was originally of type J; it produced at self-differentiation the additional type L. In six split pairs taken August 15 to 16, 1940, there was a marked difference in size between the two members. The larger member was designated *a*, the smaller *b*. One of the split pairs was lost; in the remaining, five tests showed that the larger member *a* was in each case of type L, the smaller member *b* of type J.

But persistent differences in size and form are not to be observed in all cases of self-differentiation, though they appear to be common. In many cases it is not possible to classify accurately by size or form into two mating types before tests are made. The differences in size and form are either absent or so slight as to be readily masked by differences due to other causes. The difference in size commonly observed between the two members of split pairs appears in many cases to disappear in the growth and multiplication that produces the later clones. The same clone may at one date yield split pairs that uniformly differ in size and form of the two members, in correspondence with the mating types to which they belong; at another date produce split pairs that do not show this uniform diversity. So in the clone 2H51*a*, described above, 26 split pairs showed striking and uniform differences between the two mating types, but 14 other split pairs obtained from this clone at a later date (January 21) had these differences much less clearly defined. An attempt to distinguish beforehand in 12 of these the two mating types was successful in only nine cases out of the twelve.

The differences in size and form, where they exist, seem to be indices of a constitutional difference, but this constitutional difference does not always manifest itself in this particular way.

Differences in Fission Rate.—Besides the morphological differences there were in the case of the two types produced by self-differentiation in the above clone 2H51*a* differences in fission rate. The clones from the two members (*a* and *b*) of any pair were kept side by side in small watch glasses. As set forth above, in every case the clone from one member of the pair consisted of individuals that were longer and more slender, and were of mating type B, while the clone from the other member was shorter and thicker and was of mating type C. On January 20 it was observed that in each pair of cultures, one of the two swarmed with individuals, while in the other the individuals were few. The difference was very great. Further examination showed that in every case it was the clone of longer slender individuals of type B that had multiplied greatly, while the clones of C type (short and thick individuals) had multiplied very little. In this case, therefore, the original type B multiplied more

vigorously than the derived type C. A similar difference was observed in the clones from split pairs of certain other clones in Variety I.

In the self-differentiation of clone S of Variety II, again it was the derived type L that multiplied more rapidly than the original type J. Ten split pairs were obtained July 6, 1940, *a* and *b* of each being allowed to multiply separately; later (as before noted) one of each pair was found by test to be of type J, the other of type L. On July 9, after three days, the number of individuals present was counted for each *a* and *b* clone separately. In every case the number was much greater in the clone which later tests showed to be L. The numbers present in the two clones from each of the ten pairs were as follows:

J clones: 8–8–5–4–6–7–5–11–4–10.
Mean: 6.8.
L clones: 15–17–27–28–10–17–16–27–16–16.
Mean: 19.9.

In the J clones the range of variation is from 4 to 11; in the L clones from 10 to 28. The mean is nearly three times as great for the L clones as for the J clones. In this case, therefore, the clones of the derived type (L) had at first a more rapid fission rate than those of the original type (J).

In clone NH13 of Variety II there is again more rapid fission in the clones of the derived type (E), as compared with those of the original type (H). Five days after isolation of twelve clones, four of which turned out to be H while eight were E, the numbers present in the H clones ranged from 16 to 32, with a mean of 21; while the numbers present in the E clones ranged from 44 to 72, with a mean of 58.

But as in the case of size differences, a uniform difference in fission rate in favor of one of the types is by no means always found in self-differentiation. In many cases the slight variations in rate seem distributed indiscriminately in the two types.

Differences in Readiness to Mate.—In a considerable number of cases there is a marked difference between the two types that result from self-differentiation, with respect to readiness to accomplish the mating reaction. In testing the clones to determine the mating type, members of each clone (*a* and *b* in the case of split pairs) are mingled separately with testers of each of the four (or eight) mating types of the variety. Frequently there is with all but one of these tester types an immediate reaction; clots of adhering individuals are formed from which united pairs later emerge. (The type with which reaction does *not* occur is of course the type to which the tested clone belongs.) But in some tested clones this reaction (clotting and mating) does not take place at once, but only after a period of time, which may be one to several days, during which the two clones live intermingled in the same culture.

Such a difference in readiness to react is often found between the original and the derived mating types resulting from self-differentiation of a clone. The first case observed was in the clone 2H34*b* of Variety I. This was almost certainly of the mating type B. From it, thirteen split pairs were obtained and from *a* and *b* of each, clones were derived (December 1–14, 1939). On December 10 tests were made for three of the split pairs (6 clones). By the second day of the test one member of each pair had reacted fully (with three of the tester types), while the other member had not. All those that had thus fully reacted were of the original mating type B. The other member (derived type D) of each pair did not fully react till several days later. Thus the original type conjugated more readily than the derived type.

Of the other ten pairs tests were made December 14. In six of them the original type B reacted first, while the derived type D did not react first in any case. That is, in nine of the thirteen split pairs the original type B reacted a day or more before the derived type D, while in four of them the two reacted on the same day.

Thus the animals which remain of the original type (B) are prepared to react at once. But the process by which the derived type (D) has been produced from the original type has in some way weakened the tendency to react; so that a period of a few days is required before the derived type has again attained its full reactiveness.

This difference in the two types as to the strength of the tendency to react appears most decidedly when the tests are made early, within a few days (three or four) of the time that *a* and *b* were isolated.

Other instances of a difference in readiness to react between the two types resulting from self-differentiation are the following (in all cases in which a certain clone is stated to react earlier or more readily than its mate, this means that it reacted at least 24 hours earlier; often more than 24 hours earlier).

Clone 2H54*b* (Variety I). Twenty-two split pairs, yielding forty-four clones; in each case one member is type B (original), the other D (derived). The original type B reacted first in twelve cases; the derived type D did not react first in any case. The two reacted at the same time in ten of the 22 pairs.

Clone 2H51*a*, of Variety I. Self-differentiation produced the two types B (original) and C (derived). Of the 8 split pairs obtained January 5 to 11, type B (original) had by January 22 reacted in all cases (8), the type C (derived) in but three cases. A total of 40 split pairs were obtained from clone 2H51*a*. In 29 of these there was a difference of one to several days in the time of reaction of the two members. In every one of these 29 it was the clone of type B (original) that reacted first. In the remaining 11 pairs the two member types, B and C, reacted on the same day.

It will be recalled that in this case (Clone 2H51*a*) the original type B has longer and more slender individuals, and these multiply more rapidly than the shorter thicker individuals of the derived type C. The two types produced by self-differentiation thus differ in this case in four observable ways: in mating type, in form and dimensions, in rate of fission, and in strength of the tendency to conjugate.

Clone J17*b*1 (Variety I). Twenty-nine of the sixty split pairs were tested as to readiness to react. In 25 of the 29 the original type B reacted first: the two reacted at the same time in 3 cases, while D reacted first in one case.

Clone LP10 (Variety I). Of the six split pairs in every case the member that was of the original type D reacted several days before the one that was of the derived type C.

In Variety II, the original type (H) was observed to react more readily than the derived type (E) in the clone NH13. There were numerous other clones, of Varieties I and II, in which a difference in readiness to react could not be found.

To summarize, self-differentiation of a clone gives rise to individuals which may differ in four different ways: (1) in mating type; (2) in size and form; (3) in rate of multiplication; (4) in readiness to conjugate. The only one of these that is to be observed in all cases is the difference in mating type.

In Variety I, greater length and slenderness, a higher rate of fission, and a greater readiness to conjugate belong to the original type; the derived type being shorter and thicker, slower in fission, and less ready to conjugate. In Variety II, so far as observed, the relations are the same, save that in some cases at least it is the derived type that has the larger individuals and divides more rapidly.

8. Relation of Self-Differentiation of Clones to Constitution of Wild Populations. Collections from a particular body of water often include just two mating types, such that when one of these two types undergoes self-differentiation, it produces the other. Examples of this are the following:

(1) On November 22, 1939, I received from Doctor T. M. Sonneborn a collection of *Paramecium bursaria* from a pond at Morgan, Monroe State Forest, Indiana. These were designated Mo. The animals belonged to Variety II; some were of type F, others of type M. Two clones of each of the two types were cultivated. All four underwent self-differentiation, the two F clones produced the additional type M, the two M clones produced the additional type F. Apparently, therefore, the entire population was of the constitution (FM), some being F(M), others M(F). Either could by self-differentiation produce the other.

(2) From a pool in the grounds of the Del Monte Hotel, Monterey, California, came a collection known as DM. It included only individuals of two of the types of Variety I, namely C and D; of 60 clones, 44 were C, 16 were D. One of the D clones (DM13) underwent self-differentiation; it produced the derived type C. It seems probable that the population was, therefore, of the constitution (DC), some being D(C), others C(D), either transformable into the other.

(3) From a gold-fish pool at Westwood Hills, Los Angeles, was obtained a population designated Br. The Br population included only the types B and C of Variety I. Of 30 clones, 17 were of type C, 13 of type B. One of the clones (Br48) that was of the B type underwent self-differentiation: it produced then the other type C. The constitution of the population was presumably (BC), some of the clones being B(C), others C(B).

(4) A collection obtained from a pool in the Botanical Garden of Johns Hopkins University consisted of clones of the two types E and F of Variety II. One of the clones (JH2) underwent self-differentiation, producing both E and F.

Later, the E type produced anew F, while the F type produced anew E, as before set forth.

It is however by no means always the case that a wild population consists of but two types, either transformable into the other. In the population *He*, of twelve clones, five were of type A, six of type D (and when D differentiated it produced A): but there was one clone of type B. The population Lo, from a pool at Baltimore, included representatives of all four mating types of Variety I. A population *St* from Staunton, Virginia, included representatives of four types of Variety II, types E, G, H and J. A population *Sw*, from a pool near Cockeysville, Maryland, included all the 8 mating types of Variety II.

Effects of Self-Fertilization and of Inbreeding

Clonal self-differentiation, its nature and what it reveals as to the constitution of the stock has up to this point been the center of interest. A similar examination is required for self-fertilization, particularly as to its effects on the clone or stock that undergoes it. Self-fertilization is a particular case of inbreeding; with it must be treated consequences of close inbreeding that are not precisely due to self-fertilization.

As before set forth, self-fertilization occurs but rarely in clone cultures. It is, therefore, impossible to procure from such cultures self-fertilized pairs in such numbers as are required for genetic study. This difficulty is met by the use of split pairs. If from a clone culture even a single split pair is obtained (in the way described on earlier pages), this will furnish at any time as many self-fertilized pairs as are desired. Two members of the split pair are cultivated separately, each yielding a clone. The two clones, like their parents, are of different mating types. When individuals of the two clones are mingled, they conjugate, thus providing unlimited numbers of the pairs that result from clonal self-fertilization.

Among other effects, clonal self-fertilization alters the mating types present in the clone. Inheritance of the mating type in self-fertilization and in inbreeding must therefore be first presented. In giving an account of this, only matters particularly germane to self-fertilization and inbreeding will be taken up, reserving the general phenomena of inheritance for a later paper.

Inheritance of the Mating Types in Self-Fertilization and Inbreeding

Self-fertilization has been observed, with its results so far as concerns the mating types produced, in clones of all the four mating types of Variety I. In some of the types the number of observations is few; in others it is adequate for determining the nature of the results.

Self-Fertilization in Mating Type D.

The consequences of self-fertilization have been most fully observed in clones of Type D. These are as follows:

(1) The H Stocks. The H stocks are a succession of clones and pairs all descended from the single individual He6, of mating type D. Their history illustrates particularly well the phenomena and results of clonal self-fertilization. The successive clones and pairs are the following.

H Pairs. As before set forth, on May 4, 1938, the clone He6 of Variety I (derived from a single individual of April 21, 1938) underwent self-fertilization. It was of mating type D; by self-differentiation it had produced the additional type A. The individuals of the two types then conjugated (May 4), so that the two individuals of each pair are of mating types D and A respectively. Forty-eight pairs so formed were isolated May 4, and numbered H1 to H48 (two were later discarded, so that the final number was 46). The two members of the pairs separated during the night of May 5. The two ex-conjugants were then isolated, one being designated *a*, the other *b* (this on May 6). There was no fission till the night of May 7; all the ex-conjugants then divided. There was thus a period of seventy-two hours or more from the time the pairs were isolated until the first fission. This makes it certain that conjugation had been consummated in all the pairs, since individuals that have not conjugated divide every twenty-four hours, or oftener.

The two products of fission of the ex-conjugants *a* and *b* were isolated as *a*1 and *a*2, *b*1 and *b*2. Thus from each pair four clones were cultivated, each receiving a distinctive designation, as H1*a*1, H27*b*2, and the like. The total number of clones cultured from the final forty-six pairs was thus 184. These were cultivated under favorable conditions, each in a small vessel of lettuce infusion seeded with a single species of bacterium, as described in my earlier communication (1939). In this medium, individuals that

have not conjugated flourish and undergo fission at least once every twenty-four hours.

The majority of the ex-conjugant clones flourished, and multiplied at about the rate just mentioned. But as is usual after conjugation, a number of the ex-conjugant clones after a time ceased to multiply, became abnormal or weak, pale and thin, and finally died. It is notable that usually the four clones from any pair have the same fate; if any of them weaken and die, all four do so. The four clones of a single pair are of course cultivated independently; they are no more alike in their environment than are any four taken at random. The usual identical fate of the four appears clearly due to their common genetic constitution.

By May 25, three weeks from the time of conjugation, seventeen pairs (37 per cent of all) had thus died out completely, in all their four clones. There were thus left representatives of twenty-nine pairs. Six of these had lost one or more clones, two had lost one, three had lost two, one had lost three. In the rest the four clones all survived. Thus at this time there were left 105 clones, representing twenty-nine of the original forty-six pairs. Forty-three per cent of the clones had died out.

These 105 clones remained immature for long periods, varying from a little over a month to more than two years. In time all but three became mature (these three belonged to different pairs, and in each of these there were three other clones that reacted fully, so as to show the type to which the pair belonged). The clones were subjected to frequent tests with the four testers of Variety I, until the type to which each belonged had been determined. The results are as follows:

In every case all clones descended from any pair are of the same mating type. Of the four clones from any pair, two are descended from one parent, originally of type D, the other two from the other parent, originally of type A. Yet all four belong now to the same type. From Pair 1 the four clones are all of type A; from Pair 13 the four clones are all of type D; from Pair 46 the four are of type B.

The four clones from any pair are of course cultivated separately, and this separate cultivation continues for months, before the clones are mature. The four clones of any pair do not as a rule become mature at the same time. Yet with their separate cultivation for long periods and their separate testing at different times, it finally turns out, when the results of all tests are finally tabulated, that the four clones of any pair belong to the same mating type, though those of different pairs may belong to different mating types. The common mating type of the four clones must therefore be the result of their common constitution immediately after conjugation. This common constitution might be due to chromosomal similarities brought about during conjugation. Or it could conceivably be due to certain environmental conditions operating during the time that the two members of the pair are united. In the latter case this environmental determination would have to be inherited in later fissions. In any case, it is clear that the exchange of pronuclei in conjugation has resulted in making the two ex-conjugants, that were of diverse mating types at the time of union, of the same mating type.

Of the twenty-nine pairs, fifteen gave descendants that were all of type A; thirteen gave descendants that were all of type D. Thus in twenty-eight of the twenty-nine pairs the descendants were of the same type as one of the two parents; approximately half like the A parent, half like the D parent.

But the remaining single pair gave descendants all of which were of type B. The two parents A and D give in this pair four clones that are of a different type from either.

The general result is then as follows: twenty-nine pairs, D × A, yield after conjugation fifteen pairs that are A throughout, thirteen that are D, one that is B. Of the remaining type C of Variety I none were produced.

The appearance of the type B among the descendants (though in but one pair out of twenty-nine) is of interest. It will be recalled that the original clone from which all are derived by self-fertilization was of mating type D. It differentiated, producing the additional type A, so that the constitution of the original clone could be represented as D(A). Now the two products of differentiation unite in conjugation. They produce in most cases again (as might be expected) the types A and D. But in one pair they produce the type B.

HH Pairs. Inbreeding was continued further by mating together one of the A clones and one of the D clones produced in the conjugation just described. This gave a second inbred generation of the H stock. The pairs so produced were designated HH. Four clones were cultivated from each pair, as in the first generation pairs.

The two parent clones A and D were mated September 1, 1938. From their union 57 HH pairs were obtained (September 2–September 4); the four clones from each of these were cultivated in the same way as the H pairs. In this second inbred generation mortality was much higher than in the first. By September 20, about two weeks after conjugation, 157 of the 228 clones were dead; that is, sixty-nine per cent had died. This mortality continued; by November 9, only 22 clones, representing 11 pairs, remained. When finally tested, half the clones were still immature. Eleven clones representing seven pairs were determined by tests. Five were of type A, two of type D. All the surviving clones thus in this case belong to one of the two parental types.

2H Pairs. Another second inbred generation was later obtained by mating on April 20, 1939, two more of the clones derived from the H pairs—one clone being as before of type D, the other of type A. Sixty pairs were isolated on April 24. Since it had by this time become clear that all the descendants of a single ex-conjugant are of the same mating type, only one clone was cultivated from each ex-conjugant, two (*a* and *b*) from each pair. There were thus one hundred twenty clones; these were called the 2H clones. Mortality was again high. By May 10 there remained but twenty-four clones, representing eighteen pairs. Of the sixty pairs, seventy per cent had died out completely. Of the one hundred twenty clones eighty per cent had died. Of the surviving eighteen pairs, ten were of mating type B, three of type A, two of type D; while in one pair one of the ex-conjugants gave clones of type B, the other of type A. Two of the pairs could not be determined with certainty because self-fertilization occurred in them before tests were completed.

Thus in this case, although the parents were again of types D and A, a large majority of the descendant clones were of type B. No clones of type C had as yet appeared among the descendants of the original self-fertilized D.

Self-Fertilization in Clones Descended from 2H.—The results of inbreeding were further studied through the occurrence of self-fertilization in five of the clones descended from the 2H pairs just summarized. These constituted a third generation of clone inbreeding. Each of the five was descended from a single ex-conjugant of 2H. From four of these five clones pairs and split pairs were obtained. These four were designated respectively 2H34*b*, 2H51*a*, 2H54*b* and 2H58*a*. Some account has been given of these in the section on self-differentiation. They are likewise instructive for the study of inheritance of mating type.

The clones 2H34*b* and 2H54*b* differentiated before self-fertilization into the two types B and D, while 2H58*a* differentiated into B and A. The three types A, B and D have before appeared in the H stocks. But the clone 2H51*a* produced through self-differentiation the mating types B and C. This is the first appearance of the mating type C among the descendants of clone He6, of type D. By its appearance it is demonstrated that the single type D by self-differentiation, self-fertilization, and inbreeding among its descendants, is capable of producing all the four mating types A, B, C, D, of the variety to which it belongs.

As will be set forth further in the next section, very few pairs or clones survived (to become mature), in the pairs resulting from this third step in inbreeding. Out of a very large number of conjugant pairs, a total of but twenty-six clones survived to be tested. Of these sixteen were mature: all were either type B or type D. These add nothing significant to the knowledge of the genetics of the H stocks.

To summarize, the stock H began as a single individual of the mating type D, the entire stock descended exclusively from this individual, with no accessions from outside it. This individual produced by vegetative reproduction a clone which differentiated into the two mating types A and D. These conjugated, and among their descendants were clones of all the four mating types A, B, C and D. The single type D has the potentiality of producing all four mating types.

Other consequences of inbreeding that are illustrated by the history of the stock "H" are set forth in a later section.

The DM Stock. Sixty clones were obtained from sixty individuals collected December 24, 1939. These were designated DM. In one of these clones, DM13, of the mating type D, self-differentiation occured into the two mating types D and C. A single split pair consisting of these two types was obtained January 31, 1940; the two types were isolated as two clones. On March 23, 1940, these two were mated; from them one hundred and two pairs were obtained. As usual many of these died or remained immature for many months. Up to the present time, (April 17, 1941) the mating type of thirty-one

of these pairs has been determined; fifteen were of type D, thirteen of type C, two of type B, one of type A.

Thus the single individual DM13 of type D gave origin by self-fertilization to clones of all the four different types of the variety to which it belongs (VarietyI).

Self-Fertilization in Mating Type A.

Clone 881*a*. This was derived from a cross of the two cones *l* and *m* of the mating types A and C respectively. It was itself of the mating type A. On October 19, 1937, it underwent self-fertilization. Of five pairs which it produced, three were of type A, one of type B, one of type D. Thus from a clone of type A, three of the four types of Variety I were produced.

Self-Fertilization in Mating Type B.

As shown in Table 1, different clones of the mating type B may yield at self-differentiation as the derived type either A or C or D, so that from type B all the four types are derived. But from any single clone of type B, the production of only one additional type has thus far been observed. A typical case is the following:

Clone Br48. This clone was originally of type B. It underwent self-differentiation into the types B and C. These two conjugated. Twenty-three pairs produced descendants that were tested. Of these, nine were of type B, fourteen of type C. Thus all descendants were of one of the two parental types.

Self-Fertilization in Mating Type C.

Clone *m* of mating type C. Five pairs were produced by self-fertilization in this clone. Two pairs yielded descendants of type C, while three gave descendants of type B.

Clone Wat1. This was a clone originally of mating type C, derived from a single individual of July 12, 1939. It underwent self-differentiation and self-fertilization, producing among its descendants clones of the three types A, B and C (see the fuller account in a later section).

From the above it is obvious that fully adequate observations exist only for clones of type D. But certain positive facts emerge from the observations in all four types. These are the following:

A clone of type D may yield by self-fertilization descendants of all the four types A, B, C and D.

A clone of type A may yield by self-fertilization descendants of the three types A, B and D.

Of type B, a single clone is thus far known to produce only clones of types B and C. But different clones of type B have produced in different cases A, B, C and D.

Clones of type C may produce by self-fertilization clones of the three types A, B and C.

It is possible that more extended observations will show that clones of types A, B and C may each produce all the four types that occur in the variety.

Sterility in Later Inbred Generations

As earlier mentioned, a considerable number of pairs from self-fertilized clones die after conjugation without producing descendants. In the H stocks, after the first self-fertilization thirty-seven per cent of the pairs died without producing descendants. When clones resulting from this self-fertilization were mated together (in the pairs HH and 2H), 70 per cent failed to produce descendants.

Of the clones that did survive after this second inbreeding, four underwent self-fertilization, thus constituting a third successive close inbreeding in the stock derived from the single individual He6. The pairs from these self-fertilizations were still less fertile than earlier. Their history is as follows:

The clone 2H34*b* underwent self-fertilization during the first twelve days of December, 1939. From it were obtained at that time fifty pairs, of the types B × D. All died within four days.

During the same period the clone 2H54*b* underwent self-fertilization, yielding pars B × D. Of these pairs forty-five were obtained, the two-exconjugants of each pair being cultivated separately. All the ninety clones from the ex-conjugants died within a few days, except two, belonging to different pairs. The survivors were clone 30 of type D, and clone 32*b* of type A.

January 6 to 26 the clone 2H51*a* underwent self-fertilization, yielding pairs B × C. Of these pairs, one hundred and thirty-five were isolated and cultivated in the usual way. All except two died within a few days, in most cases without dividing. The two that survived were of type B.

In the cases just described, the method of cultivation was that which was most successful in other cases, and many other clones, not derived by inbreeding, were flourishing in the same culture medium at the time these died.

This very high mortality, or lack of fertility, after three inbreedings appeared of interest, so

TABLE 3

Results of self-fertilization and inbreeding. Pairs from self-fertilized clones descended from the inbred pairs 2H. Third inbreeding in the stock.

Self-Fertilized Clone	Date of Self-Fertilization	Number of Pairs	Mating Types of the Pairs	Result
2H54*b*	Dec. 1–13, '39	45	B × D	All died but 2
2H34*b*	Dec. 2–12, '39	50	B × D	All died
2H51*a*	Jan. 6–26, '40	135	B × C	All died but 2
2H58*a*	Jan. 27, '40	48	B × A	All died
2H51*a*	Jan. 27, '40	48	B × C	All died
2H34*b*	Jan. 27, '40	24	B × D	All died
2H54*b*	Jan. 27, '40	24	B × D	All died
2H51*a*	Jan. 24–Feb. 8, '40	60	B × C	All died
2H54*b*	Feb. 9, '40	48	B × D	18 clones survived (13 pairs)

Total number of pairs 482, of which 17 survived (3.5 per cent).

that additional self-fertilizations were made, using the same methods of cultivation. The results of these are summarized in Table 3. In this table are included for completeness the three cases just described.

Only one of the self-fertilized clones, namely clone 2H54*b*, yielded pairs that survived in any appreciable number. From this clone a total of one hundred and seventeen pairs were obtained. There survived representatives of fifteen of these, so that one hundred and two of the one hundred and seventeen pairs died without coming to maturity; a mortality of 87 per cent. Also, in clone 2H51*a* there survived representatives of two pairs out of two hundred forty-three included in Table 3. In the other self-fertilized clones the mortality was one hundred per cent.

It seemed desirable to determine so far as possible whether the death of the ex-conjugants was the consequence of anything in the nutritive conditions. The organisms were living in the lettuce culture medium to which bacteria of a single species had been added, as described in my former paper (1939). It had been found that the addition of an algal culture, as described in the paper just mentioned, is unnecessary. The organisms flourish equally well without the algæ.

It was noticed however that organisms living among filamentous algæ in ponds are somewhat larger, deeper in color, and seemingly more robust than those in the lettuce medium. If filamentous algæ such as *Spirogyra* were added to the lettuce medium, it was found that in the course of a week or two the animals take on the deeper color, slightly larger size, and generally more robust appearance seen in the animals collected from ponds.

Will the inbred animals flourish better after conjugation if cultivated before and after with the filamentous algæ? This seemed a possibility, so that cultures of the inbred stocks were prepared in which this condition was fulfilled. The inbred clones were first cultivated for some weeks in lettuce infusion to which had been added filaments of *Spirogyra* or of some other filamentous algæ. The animals took on the expected deeper color, larger size, and more robust appearance. They were then mated as before. Part of the matings were of the two members of split pairs from the same clone; these were therefore pairs from self-fertilization of the 2H clones, descended by inbreeding from the clone He6. A number of matings were made also between the different inbred stocks derived from the 2H pairs. After conjugation the ex-conjugants were further cultivated in lettuce infusion containing filamentous algæ. The chief data for these matings are given in Table 4.

Table 4 shows that culture in a medium containing filamentous algæ, although it makes the individuals more robust and deeper in color, does not increase the rate of survival after conjugation in the clones that have been deeply inbred. The use of *Spirogyra* or other filamentous algæ in the culture medium was therefore discontinued.

Further experiments were made to test the results of crossing the inbred clones. Crosses were made of the inbred clones with each other, and of the inbred clones of the H stocks with the unrelated stock Br48. The H stocks came from Maryland, while the Br48 stock was collected in California. The further experiments involving comparisons between the results of self-fertilization or clone inbreeding, and outbreeding, are set forth in the following paragraphs.

Clone 2H58*a*. In Tables 3 and 4 it is shown

TABLE 4

Results of self-fertilization and inbreeding in pairs derived from self-fertilized clones of the 2H stock, when cultivated in a nutrient medium containing filamentous algae. Animals robust and deeply colored.

Mating	Date of Mating	Number of Pairs	Mating Types of the Pairs	Result
2H58*a* self-fertilized	Feb. 2, '40	38	B×A	All died
2H58*a* self-fertilized	March 11–18, '40	62	B×A	All died
2H54*b* self-fertilized	March 17, '40	48	B×D	All died but 1
2H58*a* × 2H51*a*	March 16–23, '40	51	A×C	All died
2H54*b* × 2H51*a*	March 12, '40	24	B×C	All died
H38*b*2 × 2H51*a*	March 16, '40	3	C×D	One survived

that self-fertilization in the previously twice inbred clone 2H58*a* results in the death of all the pairs produced. This clone was crossed with the related 2H clone 2H51*a* (Table 4), and again the mortality was one hundred per cent.

The clone 2H58*a* was now outcrossed twice to clones of the unrelated stock Br48. In both crosses 2H58*a* is of the mating type A. In the first outcross the stock Br48 was present as the mating type C, in the second as the mating type B. The results for all the matings of clone 2H58*a* are as follows:

Mating	Date of Mating	Number of Pairs	Mating Types of the Pairs	Result	Percentage that Survived
2H58*a* self-fertilized	Feb. 2, '40	38	B × A	All died	0
2H58*a* self-fertilized	Mar. 11–18, '40	62	B × A	All died	0
2H58*a* × 2H51*a*	Mar. 16–22, '40	51	A × C	All died	0
2H58*a* × Br48	April 10, '40	191	A × B	70 survived	36.6
2H58*a* × Br48	April 10, '40	71	A × C	69 survived	97.2

As this tabulation shows, outcrossing of the clone 2H58*a* to the clone Br48 raised the percentage of survival from 0 to 36.6 in one case and to 97.2 in the other.

Similar comparisons of inbred and outbred pairs were made for the clone 2H54*b*. In this clone mortality after self-fertilization was not in every case complete, though it was very high. The effects of outbreeding with clones of Br48 are shown in comparison with the effects of self-fertilization in the following:

Mating	Date of Mating	Number of Pairs	Mating Types of the Pairs	Result	Percentage that Survived
2H54*b* self-fertilized	Dec. 13, '39	45	B × D	All died but 2	4.3
2H54*b* self-fertilized	Jan. 27, '40	24	B × D	All died	0
2H54*b* self-fertilized	Feb. 9, '40	48	B × D	All died but 13	27.1
2H54*b* × Br48	Apr. 10, '40	72	B × C	36 survived	50.0
2H54*b* × Br48	Apr. 10, '40	72	D × C	64 survived	88.8
2H54*b* × Br48	Apr. 12, '40	108	D × B	85 survived	78.7

Thus in the clone 2H54*b*, outcrossing with the Br48 stock greatly increased the percentage of pairs that survived.

On their face the experiments just described appear to show clearly that close inbreeding greatly decreases the fertility in Paramecium bursaria. After the first self-fertilization the fertility of the stocks was in some cases relatively little impaired. In the H pairs after one self-fertilization the mortality among the pairs was thirty-seven per cent; in the DM stocks, 37.5 and 8.6 per cent respectively; in the stock of J17*b*1, however, it was 87.5 per cent. After a second inbreeding the mortality was higher; about 70 per cent for the pairs HH and 2H. After a third inbreeding the mortality in the pairs rose in most cases to 100 per cent. Outcrossing greatly decreased the mortality.

Yet the exact interpretation to be given to these facts is less certain than first appears. At the later inbreedings the stocks differ from the earlier ones in other ways besides the fact that they have been repeatedly inbred. They are

older in elapsed time; and they have been longer under cultivation in the laboratory. To both of these conditions importance has been attributed by certain authors for reducing the vigor and vitality of the stock. There are indications that one or both of them may play a role in the results. An extended study has been made of mortality after conjugation. It will therefore be best to reserve this matter for treatment in a paper to be devoted to clonal youth and maturity, age and death.

Effect of Clonal Self-Differentiation and Self-Fertilization on the Stock

After self-differentiation and self-fertilization have occurred in a clone, the clone is evidently no longer "pure"; that is, its members no longer all have the same genetic constitution. By self-differentiation part of the clone has transformed into a different mating type, and there are other changes.

Yet we have in the laboratory many cultures of clones that have been cultivated here for one to four years, and have presumably undergone self-differentiation one or more times (some are known to have done so), yet they still react as the same mating type as at the beginning. Clone *l* has been in the laboratory for four years. It originally reacted as mating type A. It is known to have undergone self-differentiation, since pairs were observed in it February 4, 1938. It still reacts as type A. Clone *m*, originally of mating type C, has likewise been in the laboratory four years, and is known to have undergone self-differentiation, since pairs were obtained from it July 14, 1937 and January 15, 1938. Yet it still reacts as type C. Similar relations exist in the clone 1*b*, produced from an ex-conjugant of June 18, 1937; it was of type D and still remains so (April, 1941). Many other clones could be cited in which the facts are parallel to those just mentioned. In spite of the occurrence of self-differentiation, the clones remain of the same mating type as at first.

On the other hand there are also in the laboratory clones which have long been under cultivation and which no longer react as they formerly did. Some cease to react at all (for a time, at least). Others when tested react as a different mating type from that which they at first evidenced. Examples will be given later.

To understand how these and other results are produced, keep in mind a clone culture in a small vessel, such as is used in the laboratory. It contains thousands of individuals that are all of the same mating type, and of similar morphological and physiological properties. They are of similar size and form, multiply at about the same rate, and show mating reactions with about the same readiness. Self-fertilization occurs in this culture. What changes does it induce?

The essential nature of the changes produced has been dealt with separately on earlier pages. Here it is their effect on the culture that is presented.

The occurrence in the clone of self-differentiation brings about of course the appearance in the culture of a number of individuals of a different mating type. The clone is no longer uniform with respect to mating type. In many cases, too, as before shown, the individuals of the new mating type differ morphologically and physiologically from those of the original mating type. The clone, originally uniform in its genetic constitution, is no longer so. This period, in which the clone culture contains two mating types, lasts but a short time. If at this time single individuals are taken from the culture and tested, some will be found to be of one type, some of the other. Thus, the clone NH13 was originally of the mating type H. A few pairs were observed in it July 6, 1940. On that date ten single individuals were taken from it and cultivated till they produced clones; these were then tested. Four were of the original type H, while six were of the newly produced type E. It is at this stage of course that "split pairs" may be obtained from the culture. If at this time the clone is tested as a single population, it will be found to react with all of the types of the variety. One of the two mating types present reacts with all but one of the testers: the other likewise with all but one, including the tester type with which the first does not react. Such reaction with the testers of all the types occurs very rarely, but if a clone is tested immediately after self-differentiation, it will be observed.

Next occurs self-fertilization; the individuals of the two mating types unite in pairs. After about forty-eight hours they separate. The exconjugants reproduce by fission, but their descendants are now immature, so that they do not take part in the mating reaction. If, as is commonly the case, relatively few pairs have been formed, this lack of reaction in their descendants will have no noticeable effect on the reactions of the culture. When tested it will continue to react as before, to three of the testers,

not to the fourth. It consists mainly of individuals of unchanged mating type, though its reactions are a little diluted, as it were, by the presence of a number of individuals that are immature and do not react. This is part of the secret of the retention by a clone culture of its original mating type reaction, even after self-differentiation and self-fertilization have occurred. Other relations play a role in this result; they are to be mentioned.

If self-differentiation has occurred very generally throughout the culture, so that two types are present in about equal numbers, then after the conjugants have separated, practically all the individuals will be immature, so that the culture will not show any mating reaction when tested. This situation arises but rarely, but it has been observed in a number of cases. Thus, during the period January 24 to 28, 1941, tests were made of 217 clone cultures of Variety II, which had been in the laboratory for periods of from several months to four years. All had formerly reacted actively and the mating type of each was known. Of the 217, ten now did not react at all. Their individuals were immature as a result of self-fertilization in the clone.

The condition of immaturity lasts for a long time—months or years; during this long period the descendants of the self-fertilized pairs do not react. In time, however, they begin to reach maturity. They now begin to take part again in the mating reaction. As shown above, different pairs give rise to descendants that are of different mating types. Two or three or even all four of the mating types may now be present in the culture that was originally made up of a single clone of a single mating type. The individuals of different mating type unite in pairs. Again we have the appearance of pairs in the single clone culture, but now the pairs are not all of the same combination of mating types, as they were at the time of the first self-fertilization in the clone. Some pairs may be of types A + B, others of A + C, A + D, B + C, B + D, or C + D. If split pairs are obtained at this stage, they are no longer of two types only; they may (in Variety I) be of any of the combinations just mentioned. The originally single clone has now taken on the constitution of a wild population.

This situation is very rarely encountered, and of course only in old clones that have been a long time in mass culture in the laboratory. I have observed it but twice. One case was in the stock Gr13 of Variety II. This was originally a clone of mating type K, derived from a single individual of February 25, 1938. Pairs were observed in the mass culture November 12, 1940, after it had been cultivated in the laboratory for two years and eight months. From it were obtained thirty split pairs. Of these twenty-nine were of K and M, while one was of M and G.

The second case was analyzed experimentally. It occurred in the stock Wat1, of Variety I. This was originally a clone of mating type C derived from a single individual collected July 12, 1939. In the mass cultures of this clone pairs were observed from December 9, 1940 to February 2, 1941: thus after eighteen months of cultivation. From these cultures 38 split pairs were obtained. Of these, 14 were a combination of A and C, 15 were A and B, and 9 were B and C. At this time therefore the cultures contained three of the four mating types of the variety; namely types A, B and C. These three combined in pairs in the three possible ways AB, AC, BC.

This case was analyzed by obtaining from the cultures new clones beginning with single individuals and keeping these clones under observation until the first self-differentiation and self-fertilization occurred. Two new clones were obtained, from the individuals S6*b* of December 19, 1940 and S13*a* of January 3, 1941. Each underwent self-differentiation less than a month from the time the clone was started, and now, as was to be expected, each produced pairs of but a single combination of mating types. The clone S6*b* was originally of the mating type B. The split pairs from it, nine in number, were all of the combination B and C. Of the clone S13*a* the original mating type had not been determined when self-differentiation occurred. The split pairs from it, twelve in number, were all of the combination A and B. Thus clones derived from a single individual by primary self-differentiation produce pairs that are all of the same combination of types, as in all cases listed in Tables 1 and 2.

When a stock contains individuals of a number of different types, as in the case of the stock Wat1, described above, the different types tend to unite in conjugation, their descendants being immature. All become thus immature, so that they no longer react, except in the case of the mating type that was in the majority. Not all of its members can find mates, so that there remain a number that have not been reduced by conjugation to sexual inactivity. These are the only individuals of the culture that react, and since they all belong to the same mating type,

the reaction of the culture as a whole when tested is that of their mating type.

But the results of self-fertilization in a clone are greatly modified by the fact, demonstrated on previous pages, that after self-fertilization a large proportion of the ex-conjugant clones die—either before or after division of the original ex-conjugant. The few that survive multiply and replace the clones that die. When these become mature the culture will upon test be found to be of the mating type that is characteristic of the clone or clones that have survived. Since the largest proportion of the new clones produced at conjugation belong to the same mating type as one or the other of the two parental types, the survivors will usually be of one of the parental mating types. There may survive but a single clone of one of the parental types, or more rarely, a single clone of one of the other types. In the latter case the clone culture in its later history reacts upon test as a different mating type from its type in its earlier history. A few examples of such results have been observed. Thus, between January 17 and 22, 1941, there were tested 128 clones of Variety I, which had been cultured in the laboratory for many months, in numbers of the cases for years. The original mating type was known for all these cultures. In this testing of January 17–22, 1941, 123 of the 128 cultures reacted as they had at first, while in five cases the reaction was changed. Two clones that had at first reacted as type A now reacted as type C. Also, three clones that at first reacted as type D now reacted as type C. The change of reaction was doubtless brought about in one of the ways above described.

In concluding this section, the changes of reaction in both varieties at the testing of January 17 to 22, 1941, may be summarized. A total of 345 clones that had been cultivated separately for many months were tested; 128 were of Variety I, 217 of Variety II. All had earlier reacted sexually in a definite way, and the original mating type of each was known. At this later testing, eleven of the cultures did not react sexually; 10 of these belonged to Variety II, 1 to Variety I. Ten of the 345 cultures had changed in reaction type. Five of these belonged to Variety I, five to Variety II. Of Variety I, two clones had changed from A to C, three from D to C. Of Variety II, five had changed from type K to type G. In this latter case the five clones had originally come from one, of type K, so that possibly the five should be considered but one case of a change of type.

Discussion of the more general problems raised by the phenomena described in the present paper is reserved until other pertinent matters have been presented in later papers.

Summary

1. As a rule all members of a single clone belong to the same mating type, since the type is inherited without change in ordinary reproduction by fission. Hence usually members of the same clone do not conjugate together.

2. But on rare occasions conjugation is found to occur among members of the same clone ("self-fertilization" of the clone).

3. Self-fertilization occurs within clones of Paramecium bursaria only at long and seemingly irregular intervals. In this it is like endomixis, so that it appears probable that the two are connected. Observation indicates that on the average clonal self-fertilization occurs once in about two thousand culture-days.

4. The number of pairs to be observed in a clone culture undergoing self-fertilization varies in different cases from one or two to a number that includes most of the population.

5. The individuals of a clone that unite in conjugation are found by testing members of "split pairs" to be of different mating types. One is of the original type, the other of a different type.

6. Thus before clonal self-fertilization occurs part of the individuals of the clone transform into a different mating type ("self-differentiation" of the clone). Clonal self-differentiation results in a pair of types, one original, the other derived.

7. Self-differentiation of any clone yields in all the pairs the same two mating types.

8. Self-differentiation of different clones of a particular mating type yield in different cases different pairs of types.

9. The combination of two mating types produced by self-differentiation of any clone is characteristic for that clone.

10. Thus any clone is so constituted that it yields at self-differentiation a particular combination of two types. Its constitution is therefore in some sense double; one clone is D(A), another D(B), another D(C) and so on.

11. In Variety I, which has four mating types, A, B, C and D, clones of each of the six possible

combinations have been observed, AB, AC, AD, BC, BD and CD.

12. The two mating types produced by self-differentiation of a single clone may each by renewed self-differentiation produce again the same two mating types. Thus each is still double in genetic constitution.

13. The two clones resulting from self-differentiation of a single clone may differ in a number of ways. (1) They always differ in mating type. (2) They often differ in size and form; (3) often in rate of fission; (4) often in readiness to conjugate. In Variety I the individuals of the clone that is of the derived type are commonly shorter and thicker, divide less rapidly, and are less ready to conjugate.

14. Populations collected from a given locality frequently (not always) consist of just two types, either of which may by self-differentiation produce the other.

15. By conjugation of the two types produced by clonal self-differentiation, additional mating types may be produced. In Variety I a single clone of type D may thus produce all the four types (A, B, C, D) of the variety. Clones originally of types A, B, or C have been observed to produce by self-differentiation and self-fertilization three of the four types that occur in the variety.

16. A considerable proportion of the pairs produced at self-fertilization are usually sterile; they die without producing descendant clones.

17. After two or three successive self-fertilizations in the same stock, usually all or nearly all the pairs are sterile.

18. A clone culture originally reacts sexually as a single mating type; that is, it reacts sexually with all but one of the mating types of the variety to which it belongs. After self-differentiation and self-fertilization have occurred in the clone, the sexual reaction of the culture may or may not be changed. The following situations occur:

(*a*) After self-differentiation, but before self-fertilization, the culture contains two mating types instead of one. It therefore reacts sexually with all the mating types of the variety.

(*b*) After self-fertilization is completed, part or all of the clone is for a long period immature, so that it does not react sexually. If but a part of the clone is immature, the reaction of the culture as a whole is the same as at first, though fewer individuals react.

(*c*) When, following self-fertilization, the exconjugant clones have become mature, the culture may contain several mating types. In time, these conjugate together and their progeny become immature, except for the "left-over" individuals of the most abundant mating type. The reaction of the culture as a whole is then that of this left-over type. This may be the same as the original type, or may be a different type.

(*d*) In most cases, the clone cultures as a whole retain their sexual reaction as of the original mating type, for very long periods. In the course of months or years a small proportion of the clones change their reaction type, in the way set forth.

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THE "MULE" PHEASANT[1]

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ABSTRACT

One Ring-neck "Mule" pheasant in the breeding season exemplified the earliest, another the most advanced stage of change from female to male sexuality and plumage. Both, like hen-pheasants generally, had bilaterally well-developed vasa deferentia and epididymides. One had many seminiferous tubules in both right and left "ovaries", not open to the epididymides, varying from infantile or quiescent tubules to those undergoing spermatogenesis to spermatids only, and probably recovering from degeneration due to back pressure. It had two oviducts (the right smaller) in conditions indicating male without female sex-hormone activation, like that in ovariotomized fowl or in castrate Night Herons injected with testosterone propionate. The other had but one "ovary" with many cyst-like cavities suggesting that oögonia and small follicles had undergone lipoidal degeneration and been leached out by reagents, and a few testicular tubules apparently too infantile to respond to gonadotropic hormones as the other's had done. Its single (left) oviduct, merely a groove anteriorly and infantile posteriorly, showed no signs of hormone activation. These "mules" appear to exemplify in pheasants phases of female intersexuality (Goldschmidt) from crossing species stronger or weaker in sex-differentiating factors in which female-differentiating factors fail in embryonic, juvenile, or early adult life, with early or later development of male plumage, head-furnishings, and spurs, and various states of gonads and secondary sexual apparatus. The unstable equilibrium may be due to wideness of cross as in gonadless and intersexual hybrid Doves. In one, somatic non-disjunction may condition dorsal and ventral distribution of female and male feathers. The intersexual gonads and oviducts are responsive to seasonal cyclic gonadotropic stimulation and become active in the breeding season when not too infantile.

INTRODUCTION

BISSONNETTE (1940) described the plumage features and reproductive organs of a "mule" pheasant, properly preserved for histological study, in January, and the sex-glands of another found dead in October. These were compared with homologous parts from a normal hen-pheasant preserved at the same time as the January "mule".

Macroscopically the sex-glands of both "mules" were ovaries in autumn and winter condition of regression. Histologically neither exhibited any eggs or ovarian follicles. In both, one or more regions might be interpreted as regressed follicles, as recovery sites or scars from cyst-like foci of infection, or from degenerated seminiferous tubules. They were pigmented like normal ovaries but more heavily.

The properly fixed January gonad exhibited coiled seminiferous tubules in various regions containing what appear to be spermatogonia and Sertoli cells arranged about the periphery of the tubules like those of male pheasants in January. The October gonad was so poorly preserved that fine histological structure could not be distinguished. In both birds, the paucity of proper ovarian tissues accounts for the "male" feathers of the mixed plumage; and the testicular tissue in one of them for the maleness of head furnishings and the slight enlargement of spur rudiments.

It was not known whether these birds had shown the mixture of male and female feathers and secondary sex-characters from hatching or had acquired the "male" characteristics after exhibiting completely "female" characters and behavior. So the question was left open as to whether they were intersexes or sex-inverts.

The question was raised as to whether these testicular tubules, in a gland otherwise ovary-like in structure, could respond to environmental conditions in the breeding season, undergo spermatogenic activity, and activate the oviduct and the vasa deferentia (two of which are present in the normal hen-pheasant, as well as in the "mules") and cause the assumption of the breeding phase of head furnishings characteristic of the cock. The search for other "mules" was continued in the hope that they might aid in settling these problems.

Continued interest in this anomaly by A. G. Csech, of the Game Warden Service of the State Department of Fisheries and Game, and of pheasant breeders, brought us two new specimens in good living condition, except as stated. One was more male-like in plumage and spurs than the earlier ones and was said to be mixed in feathering from the start. The other, less male-

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like, was undergoing change from almost completely female feathering toward that of the male. They are reported here because their histories are better known and dissimilar, and they consequently throw more light on the problems mentioned above.

Material and Method

One of these "mule" pheasants (no. 3 in our series) was given us by Fred. Roach, pheasant breeder, of Simsbury, Connecticut, on April 19, and sacrificed for study on April 23. It was the most male-like of those so far seen; was in full feather and almost complete breeding plumage (Figs. 1, 2, 3, 4, 5).

The other, no. 4, was the least male-like "mule" we have seen; but definitely a "mule" when secured from the Bushy Hill Game Farm, Simsbury, Connecticut, on May 20. It had been kept in a pen of chicken wire and its frequent attempts to fly through the wire, when frightened, had worn off the feathers, skin, and flesh from the top of its head, leaving the skull bare and dry over an area of about three- quarters of a square inch (Figs. 7, 8). In spite of frequent tearing open of the wound, the bird lived in this condition for more than six weeks, until killed for study on June 11.

The complete urogenital systems, keeping both sides together, and the thyroids were removed from each "mule" immediately after skinning and fixed in Bouin's fluid. Kidneys, adrenals, ovaries, oviducts, epididymides and vasa deferentia to the cloaca were included. The whole system of no. 3 was photographed after washing out the fixative (Fig. 9), divided sagittally and across the middle, and each side sectioned serially transversely at 10 microns. The two sides of no. 4 were folded so that they faced each other and sectioned transversely in one series. Photomicrographs at suitable magnifications of representative sections from each bird were made after iron-hematoxylin and faint eosin staining.

Observations

"Mule" No. 3

External Characters

No. 3 was hatched in spring, 1939, a cross of Mongolian with Ring-neck or Black-neck; Mongolian characters, such as light iris, predominated. It had a crossed bill, was wild and fairly pugnacious. The feathers on crown, nape, back of neck, shoulders, back, dorsum of tail and both wings were predominantly of female type. Consequently it lacked the two small tufts of feathers or "ears", erectile in the breeding male (Figs. 1, 2, 5). The rest of the body and the under parts of the tail were mixed male and female or predominantly male (Figs. 3, 4). The legs were small, like those of females, but the spurs were more highly developed than in the other "mules" or in normal females (Fig. 6). In size it was intermediate and nearer the male. Its behavior toward females was not noted. It was said to have given the male mating call.

Sex-organs

Its gonads bore no resemblance, in gross, to testes. On the left side was an ovary-like body with nodules, like small ovarian follicles in appearance, but fewer in number and not graded in size like those of a normal ovary. Another, similar, but smaller, narrower body on the right was nearer the mid-line. The left oviduct began as an infundibulum posterior to the "ovary" and lateral to the kidney, and extended to the cloaca with a marked fluid-filled swelling at the posterior ends of the kidneys (Fig. 9). A similar, but smaller, shorter, right oviduct exhibited a smaller fluid-filled enlargement. Both infundibula were delicate and difficult to discern in gross, but plain in sections.

Sections show that both right and left gonadal rudiments have developed and have been activated. They lack ova and follicles. Each consists of a stroma, pigmented like a normal ovary, in which coil several seminiferous tubules actively undergoing spermatogenesis with all stages up to and including spermatids, but not spermatozoa. Mitotic figures are numerous (Figs. 10–13a). In the left "ovary" is a cyst-like reticulum of cells with long processes and large vacuoles from which lipoidal substances have probably been dissolved out by technical reagents (Figs. 14, 15, 16). It contains no recognizable germ-cells. In some respects, it resembles degenerated mammalian corpora lutea, with degranulated cells and lipoidal products dissolved by reagents. But it seems more probable that it is a blind degenerate seminiferous tubule. It is the only structure in either ovary that would admit of interpretation as derived from a follicle or as representing remains of female germ-cells.

The testicular tubules of both gonads end blindly without connection to the epididymal

tubules and vasa deferentia. Some have lumina, some have not. They vary in condition from winter resting or infantile ones like those of the winter "mule" (Bissonnette, 1940) to those with almost breeding activity. Nests of germ-cells forming sex-cords rather than tubules are present and the germ-cells in them undergo spermatogenesis while in such positions (Figs. 12–13a).

Small lymphocytes have invaded some regions of both gonads and the left epididymis and vas deferens (Figs. 10, 11, 11a). The invasion is so great in some places that the tissues seem to be lymphatic in nature and in the process of developing into lymph nodes. All stages from small to large lymphocytes, and mitotic figures of dividing large lymphocytes are found in various places. In some, small lymphocytes invade the epithelial walls of the epididymal tubules and appear in the lumen. It is suggested that in some places they have cleared up or were clearing up degenerate tubules.[2] None of these regions occur in the right epididymis or vas deferens. Along the left they appear near the kidney, but not along the oviduct, in the mesentery of which the vas courses after leaving the wall of the ureter posterior to the kidney.

The left oviduct begins anteriorly as an open ostium just behind the "ovary" and extends to the cloaca. The endometrium of its distended region is not thickened nor activated as it would be by action of female sex-hormone. The right oviduct begins blindly somewhat posterior to the left. Although smaller it, also, is dilated with fluid and its wall resembles that of the left. The right vas deferens bears the same relation to the right oviduct as the left one does to the left oviduct.

Conditions of both oviducts (Figs. 9, 17) resemble those induced by testosterone propionate in castrate Black-crowned Night Herons and not those induced by estrogens (Noble and Wurm, 1940). It is worthy of note that Domm (1932) found distended oviducts in ovariotomized fowl and occasionally in those otherwise normal. This suggests that the small amount of gonadal material was secreting enough male hormone to cause the typical enlargement and distention of the two oviducts, while female hormone was scanty or wanting.

[2] We are indebted to Professor James E. Kindred for interpreting these lymphatic or lymphocyte-invaded regions and pointing out significant conditions there.

"Mule" No. 4

External Characters

No. 4 was much less cock-like than no. 3. It possessed the red region around the eye and the dark greenish black upper neck feathers. A small white neck ring incomplete dorsally and ventrally signified maleness. Because the top of its head was denuded to the bone, it was not possible to know what its condition otherwise would be. Male feathers and some intermediates were scattered among the predominantly female feathers on the rest of the body (Figs. 7, 8). A careful study of the plumage is deferred to a later time.

The bird was small, even for a female, and its spurs little, if any, larger than those of normal hens (Fig. 6). It was certainly undergoing change from female to male plumage and secondary sexual characters. Its behavior toward hens was not noted except that it dominated those kept with it since it began to assume other male characteristics. This is in accord with raised dominance in the peck-order of fowl injected with male sex-hormone (Allee, Colias and Lutherman, 1939) and with common observations on crowing hens in the barnyard, which are subservient to cocks, dominant to hens.

Sex-organs

No right gonad nor oviduct was found, nor any trace or rudiment from which either could develop. Remnants of the mesonephros formed well-defined but infantile epididymides on both sides with vasa deferentia leading to the cloaca (Figs. 18–23). These were much coiled anteriorly as far back as the posterior ends of the kidneys; closely related to the adrenals anteriorly and to the ureters posteriorly (Figs. 34–41). In some places are lumina containing colloid (Figs. 19–23), in others no colloid, and no lumina in others. They seem to be about equally developed on the two sides. Their linings failed of complete differentiation, and they end near the gonad (Figs. 18, 20). Among typical epididymal tubules are found some resembling testicular tubules (Figs. 20, 23).

Near the left adrenal and the ganglia in front of it was an infantile left ovary, with shallow cortex and flattened medulla, typical, except for the apparent complete absence of oögonia. Sections showed, in their stead in the cortex open sockets or cysts of all sizes from those barely

containing early germ-cells migrating inward from the germinal epithelium to those of partly grown follicles. These appear to be larger and more numerous posteriorly. They suggest a failure of germ-cells of female type to pass beyond the early stages of oögenesis before degenerating, perhaps from failure at synapsis or at some other stage of the growth phase. Posterior ones progressed further toward follicle-formation before succumbing (Figs. 18, 20, 24, 25, 26, 27).

The stroma is pigmented as in the normal hen-pheasant and in other "mule" gonads (Bissonnette, 1940). Primary and secondary sex-cords can be recognized in the medulla and in regions adjacent to the vacant cysts. In some places, particularly anteriorly, they contain tubules resembling those found in infantile testes, with cells resembling spermatogonia and partly activated Sertoli cells. They occur in all parts of the gonad, even farther forward than the vacant "oögonial sites" (Figs. 25–28). Regions resembling degenerated or cystic testis tubules also appear (Fig. 26). Lymphocytic infiltrations occur in the gonad and left epididymis like those in no. 3, but in fewer places (Figs. 20, 25, 26).

The histological picture suggests that, after the female germ-cells had developed to varying degrees they died off and degenerated into lipoidal cysts or vacuoles of various sizes from which the lipoids were leached out in the microscopical techniques of fixation and those leading to sectioning. Other germ-cells may have become spermatogonium-like and formed tubules of testicular type. This condition resembles that found in no. 2 "mule", but with fewer seminiferous tubules, and with vacant spaces for oögonia and follicles, not seen in no. 2. Loss of follicles may account for loss of female characters and for change over toward male plumage, very incomplete in this bird but more advanced in nos. 2 and 3. Paucity of testicular tissues and interstitials may account for the relatively slight development of male characters. The small size of the bird points to retarded development of the soma accompanying that of germinal tissue, resulting from some sort of incompatibility. No. 4 probably represents the earliest stage in a process of waning femaleness and waxing maleness among the four birds so far studied, with nos. 2 and 3 following in order of completeness of change-over.

Near the anterior end of the right adrenal and near a ganglion posterior to it, to the right of the abdominal aorta, were two or more nodules resembling testicular tissue enclosed in a tunica propria. They are about 225 microns or less in diameter and are buried in the adrenal or in tissue adjacent to a ganglion medial to the epididymis. They may perhaps be clumps of aberrant adrenal tissue.

As shown in sections the left oviduct begins anteriorly as a pair of ridges on the ventral surface of the kidney posterior and lateral to the gonad (Fig. 29). A short distance caudally it flattens to a broad low ridge covered by a loose membrane formed by the peritoneum covering the kidney (Fig. 30). A groove then appears in its ventro-lateral margin, deepens, and is followed by a second groove in its medio-ventral margin. Both grooves deepen and their lateral walls elongate and converge toward each other over the grooves but fail to close ventrally to form a duct or tube (Figs. 31, 32, 33). The whole slit-like structure remains covered in by the peritoneum covering the kidney acting as a capsule in the form of a long narrow pocket. Posteriorly the flap-like sides of this oviducal groove thicken to resemble the floor in thickness and almost close in to form a duct (Fig. 33). It resembles an oviduct slit open lengthwise (Figs. 34, 35), and assumes the proportions and lining typical of an infantile oviduct, leads off from the kidney on a broad thin mesentery, the mesotubarium, while still retroperitoneal (Fig. 33). Much farther caudally the oviducal groove closes at about one-quarter of the way short of the edges of the margins of the flaps which form its sides (Fig. 36). It thus acquires a lumen but it still leaves a deep groove externally opposite to the mesotubarium (Figs. 36, 37). Much farther back this external groove or slit closes solidly and the inner surface of the duct changes and lands and grooves appear in it (Figs. 38, 39). There remains a broad ligament or sheet of tissue extending ventrally from the surface of the oviduct opposite to the mesotubarium, and about as broad as half the diameter of the oviduct. This sheet gradually narrows and thins out so that the oviduct comes to resemble a normal infantile one posteriorly (Figs. 40, 41). The sheet or flap thickens again near the cloaca and contains some peculiar spongy tissue (Fig. 39). The whole structure exemplifies a very infantile and rudimentary oviduct, restricted to the left side.

Discussion

The gonads of "mule" no. 3 show that, in some cases, the seminiferous tubules of the "ovary"

of the "mule" pheasant undergo active spermatogenesis during the breeding season. In this bird they were evidently activated to spermatogenesis early in the season, although no mature spermatozoa were evident at autopsy. There is strong probability that either earlier or later in the season spermatogenesis may reach completion. Sojourn of the bird for some days in a shady basement room before sacrifice may have induced some regression from complete activation. Pheasants are very susceptible to photoperiodic sexual stimulation (Bissonnette, 1939 and previous papers). The absence of matured sperm from these gonads need not preclude the possibility of previous complete spermatogenesis. Necropsy may have occurred during one of the periodically recurring waves of pressure degeneration which alternate with periods of activation of germ-cell elements, known to recurr in testis grafts, after ligation of vasa efferentia, or in naturally occurring absence of vas deferens and epididymis (Pezard, Sand and Caridroit, 1924; Caridroit, 1926; Greenwood and Crew, 1926; Greenwood and Blyth, 1930; Moore, 1931). Differences in stages of activity or recovery in different parts of this "mule's" gonads lend support to this idea.

The change toward maleness of plumage with greater degree of male feathering in those "mules" with less ovarian or more testicular tissue strongly indicate a parallel change in these related structures, in line with Benoit's (1929) arrangement in series of physiological activations of secondary sexual characters. The presence of two oviducts in no. 3 "mule" and their type of enlargement and distention are due to enhanced production of male sex-hormone without the hyperplasia of their lining mucosa which would be induced by female hormone (Noble and Wurm, 1940). Such distention often appears in ovariotomized fowl and more rarely in those otherwise normal (Domm, 1932).

The development of both right and left gonad-rudiments of no. 3 into irregular ovary-like testes lacking ovarian follicles or eggs, but with greatly distended seminiferous tubules, is associated with the characteristically female loss of connections of mesonephric epididymal tubules from vas deferens to seminiferous tubules. Therefore gonad-rudiments of both sides may be affected simultaneously. Conditions in no. 4, on the contrary, show that sometimes the sex-gland and oviduct of only one side may develop accompanied by vasa deferentia and epididymides on both sides. This probably depends upon the time and stage of development at which female differentiating factors begin to fail and male factors attain ascendance.

The failure of some tubules in no. 3 and of all in no. 4 to undergo activation to spermatogenesis, the presence of tubules in intermediate condition, and the spermatogenic activity of germ-cells in some sex-cords not yet organized into tubules, in no. 3, indicate that not all gonads nor all parts of the same gonad are equally susceptible to activation and that germ-cells need not be organized into tubules before being ready for spermatogenesis. There is also a suggestion that the seminiferous tubules in no. 4 may have been at too infantile a stage of development to respond to gonadotropins if they were present in normal quantity or potency.

In no. 3, the region of the left gonad nearest to the mid-line of the body was least activated, while almost, if not quite all of the right, usually rudimentary, gonad appears to have been susceptible to activation into spermatogenesis. This right one was shorter and narrower than the left. In no. 3 there is no question of unilaterality of the condition except for difference in size of gonads and oviducts. In no. 4, on the contrary, the primarily "female" sex-organs are unilateral, without recognizable remnants on the right side. "Male" ducts are bilateral in both "mules", as in normal pheasants of both sexes, and apparently bilaterally symmetrical in development and preservation.

In no. 3, the apparent dorsal distribution of the more female parts of the plumage from top of head to tail, including most of the wings, with maleness ventral, might suggest somatic non-disjunction as an accompanying anomaly. This, however, cannot be the cause for the persistence of two oviducts and gonads, not found in normal hen-pheasants. Evident lack of dominance of either male or female type of gonadal tissue or insufficient dominance of male tissue was not associated with arrested development of the soma in no. 3. It was in no. 4, which was evidently at a much earlier stage of the process of sex-reversal or female failure. This is attested by vacant places for oögonia, fewer testicular tubules, smaller, very rudimentary oviduct, failing to close anteriorly, less male feathering, and lack of spur development. It therefore exemplifies the earliest stage of such reversal with no. 2 intermediate and no. 3 farthest advanced of the three "mules" studied so far.

The single gonad of no. 4, very markedly, and those of no. 3, slightly, resemble those found in hybrids from wide crosses in Doves (Riddle and Johnson, 1939), in which a single small juvenile ovary is found, with shallow cortex, lacking oögonia in late stages of development, although these gonads may have had germ-cells earlier in the life of the birds. This condition was ascribed to an undescribed type of sex-reversal. Vacant spaces, left by disappearing oögonia or follicles, were not found in the Doves. The spaces found in no. 4 "Mule" seem to fit well with the theory of failure of female germ-cells at some phase of oögenesis and continued development of those of male type into seminiferous tubules. The apparent repression of the female sex in the Doves was ascribed to the wideness of the cross which in some cases led to gonadless birds. Conditions in these pheasant "mules" may also be due to hybridization. The Ring-neck pheasant is a hybrid between *Phasianus torquatus* and *P. colchicus* or *P. mongolicus* or all three. The number and frequency of these intersexes of sex-inverts among pheasants is much less than among the hybrid Doves described, but the crosses are not so wide.

Bullough (1940) has just described a somewhat similar change in some old female minnows (*Phoxinus lævis* L.) which appear to undergo sex-reversal to males in many respects. He concludes that the germ-cells in the female portion of the ovaries fail, the glands cease to secrete estrin, and testicular tissue makes its appearance and develops. The male hormone subsequently secreted causes final breakdown and elimination of ovarian tissue. This is followed by reversal of secondary sexual pigmentation. In our pheasants, failure of female elements occurred relatively early, in juvenile or early adult life, and male tissue took over to varying degrees, leading to apparent sex-reversal, or, perhaps more properly, to female failure and male usurpation of function following an original bisexual condition in which femaleness first predominated.

The disappearance of germ-cells or follicles in no. 4, leaving cyst-like cavities, indicates some incompatibility of cytological elements as the cause of failure of oögenesis during the growth phase and possibly at synapsis. This may be due to wideness or complexity of hybridization with alien male pronuclear substances failing to achieve compatibility in the cytoplasm of the maternal species at the critical period during synapsis. The time when this incompatibility reaches a climax and factors for femaleness fail or lose control of differentiation and allow male differentiating factors to assume control, varies in different birds from different crosses of parent species. This resembles the type of reaction postulated by Goldschmidt (1931) for crosses between strong and weak races leading to intersexual females coming to exhibit male characteristics progressively later with weaker male strains in the cross or as the epistatic minimum for complete sex-expression falls short of realization.

It therefore appears that the "mule" pheasant is not due to the overpowering of an originally definite female sexuality by intervention of male hormone as in the cases of chicks from eggs injected with male hormone (Kozelka and Gallagher, 1934; Willier, Gallagher and Koch, 1937; and others cited by them) nor of sinistral ovariotomy allowing for temporary testicularization of the right gonadal rudiment (Benoit, 1923; Zawadowsky, 1926, Domm, 1927; and others). Those cases revert, in time, to their original genetic sex after transitory masculinization of plumage and other secondary sexual characters. The "mules" do not revert, so far as we know, but increase in amount of masculinization. They appear, therefore, to be due to inherent genetic weakness of female sex-differentiating factors which finally fail and permit hitherto almost repressed or masked inherent male differentiating factors and gonadal tissue to assume hormonal control of the soma, particularly the feathers, spurs, and head furnishings. The gonadal tissue itself becomes testicular and even undergoes seasonal breeding activity and spermatogenesis in some cases, perhaps not all.

Consideration of the above facts and generalizations of other workers may leave it still an open question whether this phenomenon should be regarded as sex-reversal or as intersexuality arising from incomplete dominance of female sex-differentiating factors in the sense of Goldschmidt. The soma appears to have been female in development up to various stages in different individuals in parallel with the gonads. Female factors appear to have lost their potency or run out and maleness and then male hormones to have assumed various degrees of control of further differentiation. It is concluded that the evidence favors a theory of intersexuality.

Summary and Conclusions

1. General feathering, gonads, and secondary sexual organs of two Ring-neck "mule" pheas-

ants, sacrificed for study during the breeding season in May and June are described. One, no. 3, is in the most advanced, the other, no. 4, in the earliest stage of change over from female to male sexual condition and plumage.

2. Both have bilaterally well developed epididymides and vasa deferentia ending blindly anteriorly, as do hen-pheasants.

3. No. 3 had many seminiferous tubules in both right and left ovary-like gonads, ending blindly without emptying into the epididymides, in different stages from infantile or resting winter tubules to distended ones with germ-cells undergoing spermatogensis to spermatids, but no sperms. The tubules were probably in various stages of recovery from pressure degeneration, such as are found in other animals with exit of sperms blocked near the gonad.

4. No. 4 had but one "ovary" containing many cyst-like cavities suggesting that oögonia and small ovarian follicles had undergone lipoidal degeneration and been leached out by technical reagents, together with a few developing seminiferous tubules apparently too infantile to be activated by gonadotropic hormone in this, the breeding season, as those of no. 3 had been.

5. No. 3 had two unequal oviducts, the right the smaller, both in conditions indicating activation by male sex-hormone without female hormone effects, similar to that induced in castrate Night Herons by testosterone propionate or found in ovariotomized fowl.

6. No. 4 had but one oviduct very incomplete and rudimentary anteriorly, as a groove, and tubular and infantile posteriorly, showing no signs of male hormone activation as yet.

7. The findings suggest that these "mules" exemplify in pheasants various conditions of female intersexuality, in the sense of Goldschmidt, resulting from crossing species stronger or weaker in male and female sex-differentiating factors, with female differentiating factors failing in embryonic, juvenile, or early adult life. This leads to early or later development of maleness of plumage, spurs and head furnishings and to various conditions of gonads and secondary sexual apparatus.

8. The wideness of the crosses may be a factor in this unstable equilibrium, as it appears to be in gonadless and other intersexual hybrid Doves.

9. No. 3 "mule" may have somatic nondisjunction as an added factor leading to dorsal distribution of female and ventral distribution of male feathering.

10. The active spermatogenesis, producing spermatids, in the testis tubules of no. 3 "mule" demonstrates that these intersexual gonads are responsive to the cycle of gonadotropic hormone production with the seasons, when not too infantile, and become active in the breeding season.

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PLATE I

FIG. 1. Left side of "Mule" Pheasant, no. 3, to show ring-neck and dorsal distribution of "female" feathers and ventral "male" feathering.

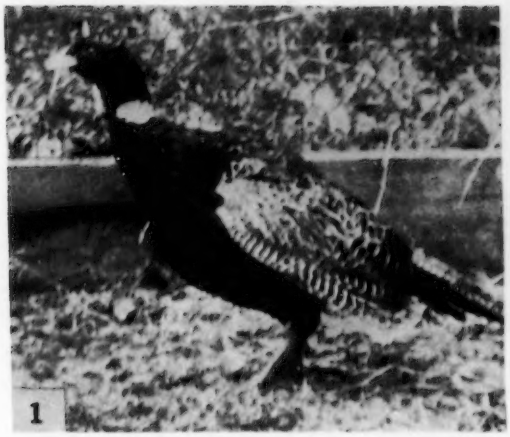
FIG. 2. Right side of "Mule" Pheasant, no. 3, as above.

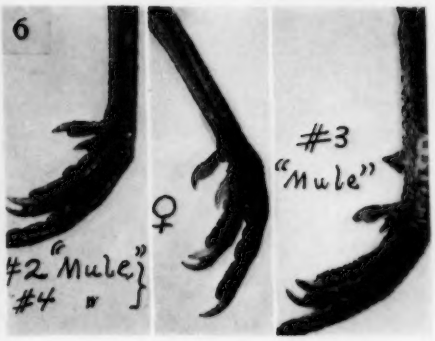
FIG. 3. Ventral view of "Mule" Pheasant, no. 3.

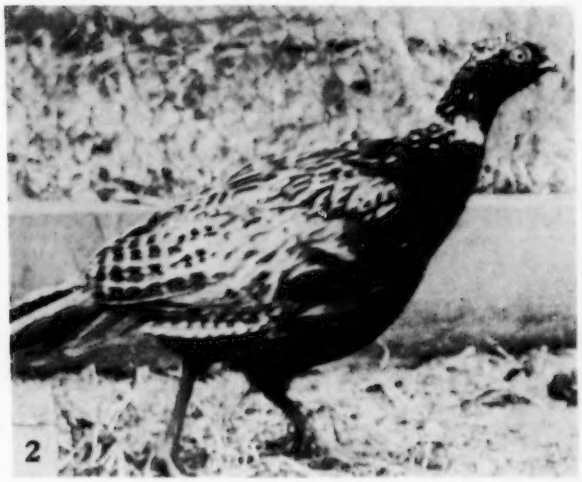
FIG. 4. Ventral view of "Mule" Pheasant, no. 3, to show red ring around the eye and male ventral feathering.

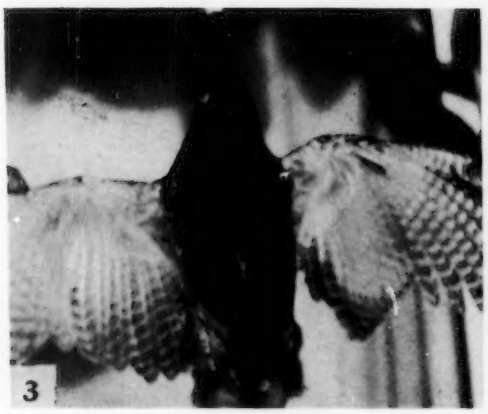
FIG. 5. Dorsal view of "Mule" Pheasant, no. 3, to show female feathering.

FIG. 6. Photographs of legs of "Mule," no. 2 or 4, normal female, and "Mule," no. 3, to show relative spur development.









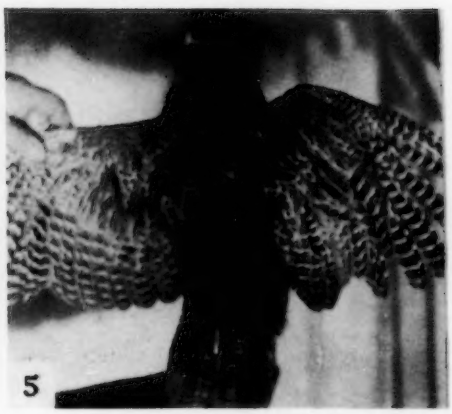


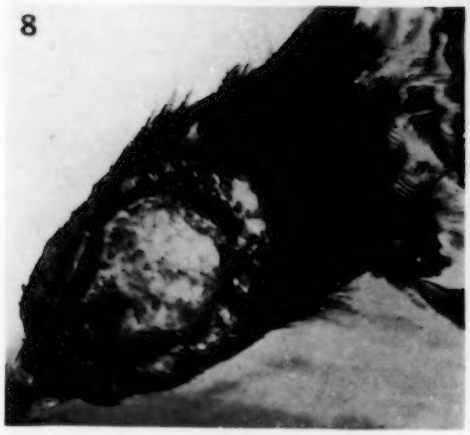
PLATE I

PLATE II

FIG. 7. Side view of head and neck of "Mule" Pheasant, no. 4, to show ring-neck, red area around eye, and denuded skull.

FIG. 8. Dorsal view of head and neck of "Mule" Pheasant, no. 4, to show denuded bare dry skull and granulation zone around it, and dorsal part of white ring on neck.

FIG. 9. Photograph of ventral view of reproductive system of "Mule" Pheasant, no. 3, showing two oviducts, right and left gonads lobulated like ovaries and their relations to the kidneys.



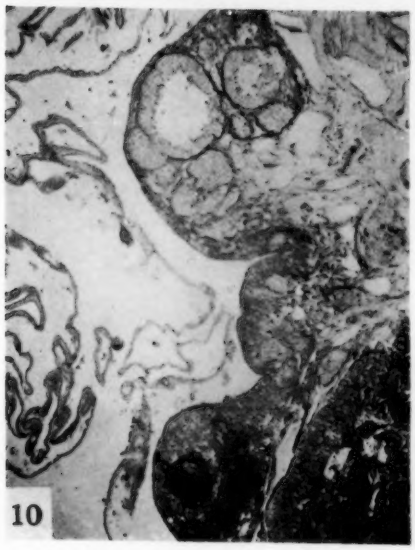


PLATE II

Plate III

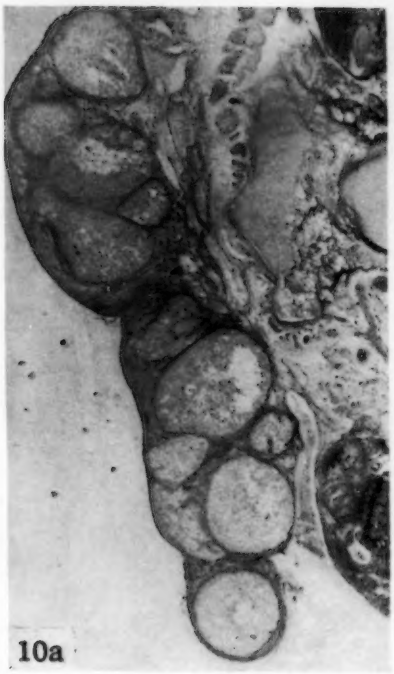
Fig. 10. Photomicrograph of cross-section of anterior end of left gonad (or "Mule," no. 3) to show seminiferous tubules, cortex and medulla of "ovary," pigmentation, and lymphocytic infiltrations. ×34.

Fig. 10a. Cross-section of left gonad about its middle. Shows epididymis in lower right corner with lymphocytic invasion. ×34.

Fig. 11. Cross-section of left gonad showing various conditions of spermatogenesis in blind tubules. ×84.

Fig. 11a. Another section of left gonad of "Mule" Pheasant, no. 3. ×84.

Fig. 12. Section of right gonad with epididymis in lower left corner. ×34.



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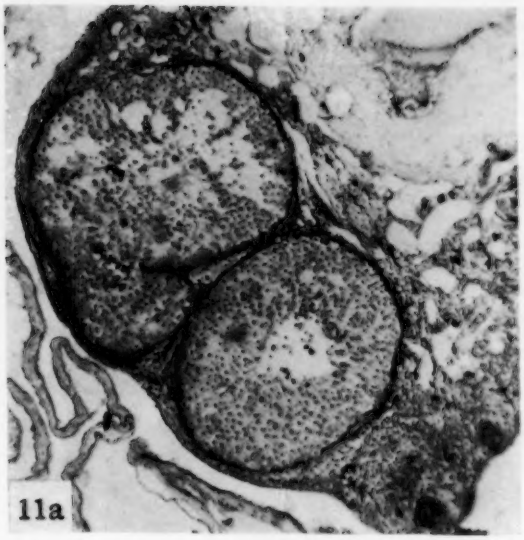

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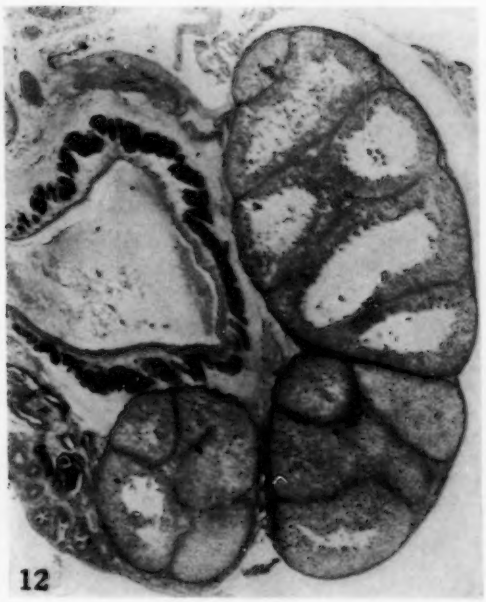

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PLATE III

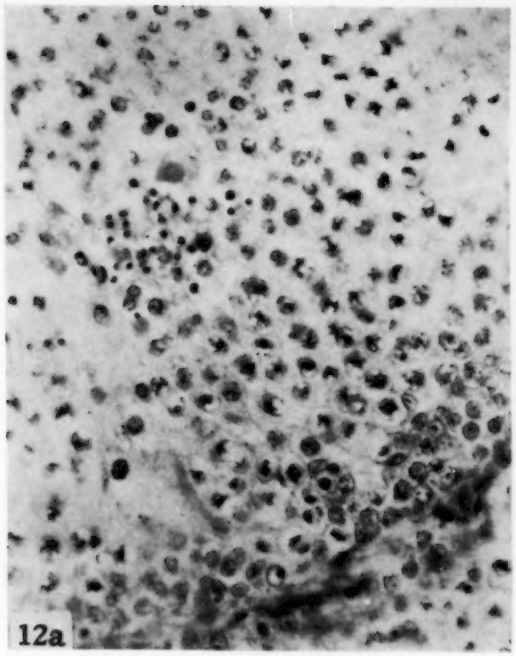
PLATE IV

FIG. 12a. Section of right gonad; seminiferous tubules. ×335.

FIG. 13. Section of right gonad. ×84.

FIG. 13a. Section of an interstitial region and adjacent tubules of right gonad enlarged from Fig. 13. ×335.

FIG. 14. Cross-section of left gonad to show large cyst-like region, probably a degenerate seminiferous tubule, and active seminiferous tubules in various stages of spermatogenesis. ×34.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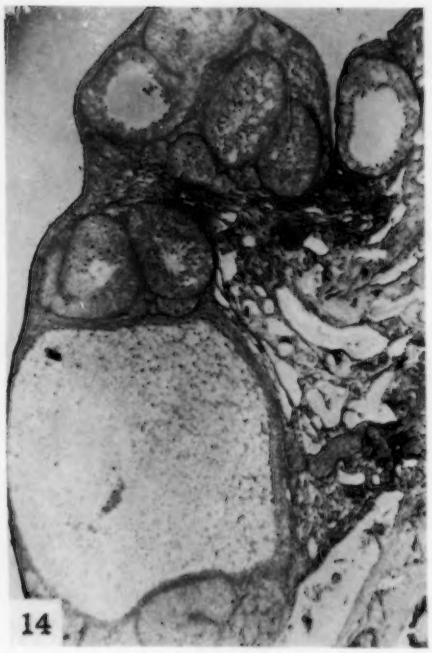
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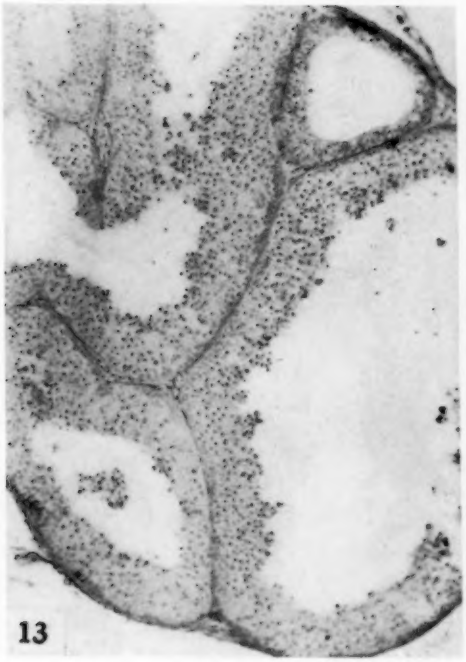
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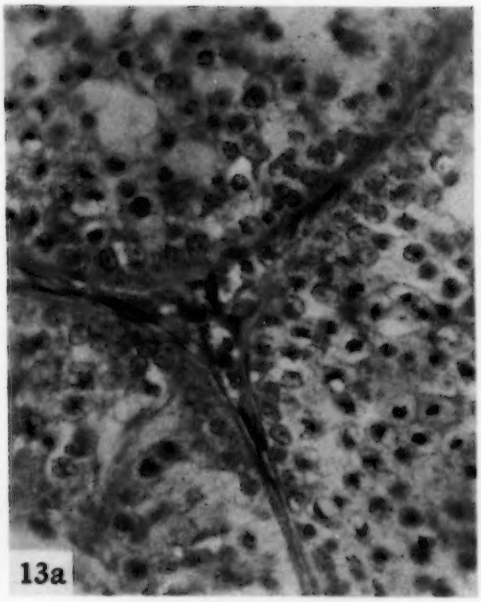
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PLATE IV

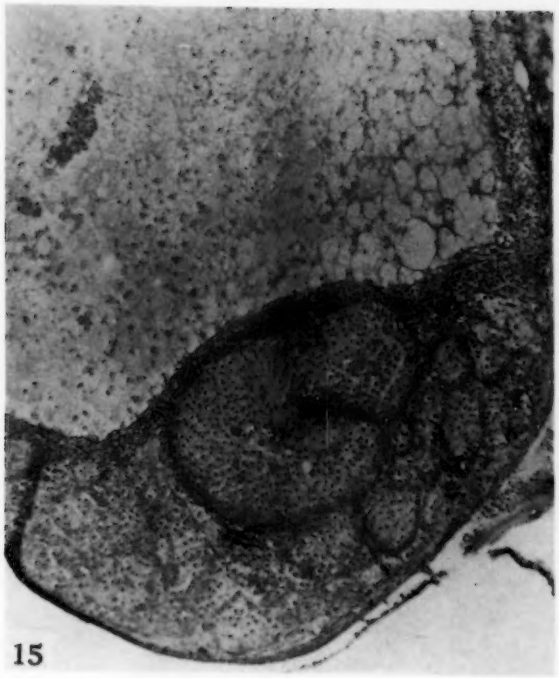
PLATE V

FIG. 15. Section of Fig. 14, enlarged to ×84, Part of cyst-like region and adjacent active tubules.

FIG. 16. Section of cyst-like region and active tubule of left gonad. ×335.

FIG. 17. Cross-section of typical left oviduct of "Mule" Pheasant, no. 3. ×34.

FIG. 18. Cross-section of left gonad and epididymis of "Mule" Pheasant, no. 4, to show vacancies where follicles have apparently been eliminated, some seminiferous tubules in infantile condition and pigmented medula and cortex. ×84.



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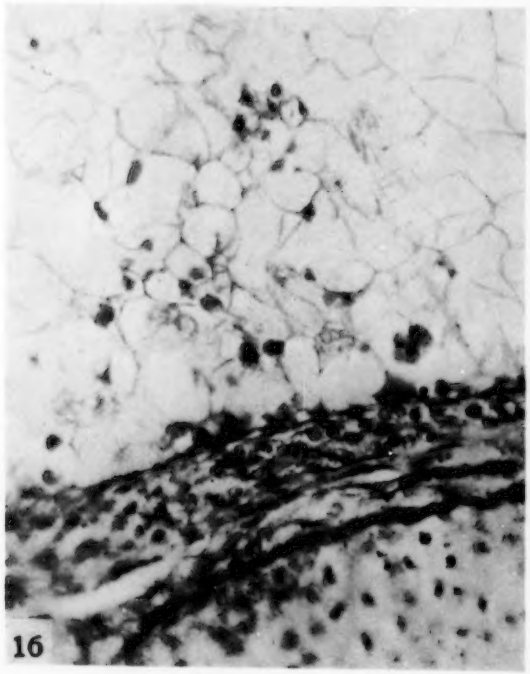

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PLATE V

PLATE VI

FIG. 19. Cross-section of left epididymal tubules to show colloid in some tubules and others vacant. ×335.

FIG. 20. Cross-section of left gonad and epididymis of "Mule," no. 4, near the posterior end of the gonad to show some tubules in the epididymis similar to those in the gonad and some fairly typical epididymal tubules. ×84.

FIG. 21. Cross-section of right epididymis at same level as Fig. 22. ×84.

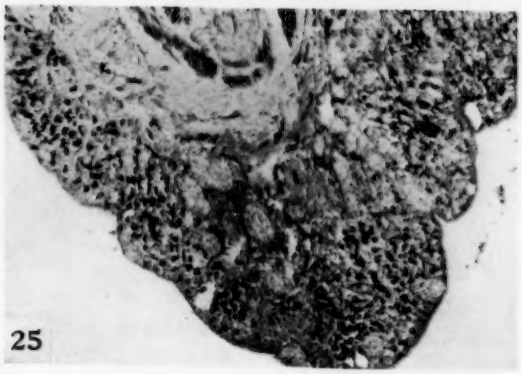
FIG. 22. Cross-section of left epididymis of "Mule," no. 4, at the same level as Fig. 21 for right one. ×84.

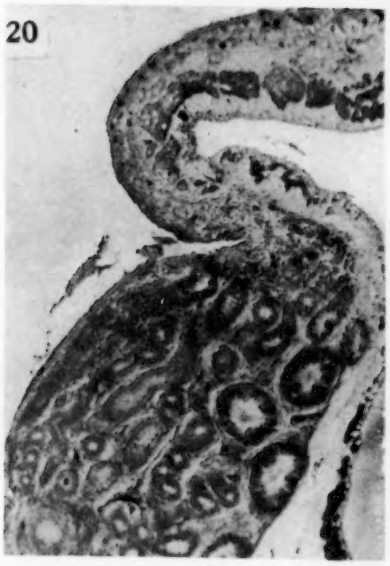
FIG. 23. Cross-section of left epididymis of "Mule," no. 4, to show tubules in it like those in the gonad among normal epididymal tubules. ×84.

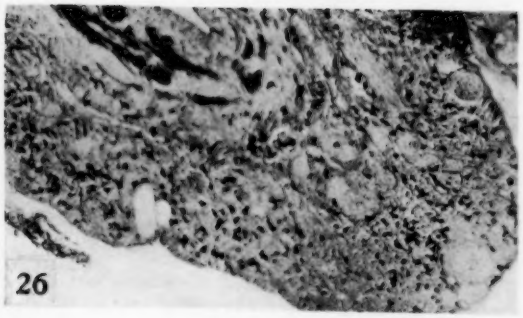
FIG. 24. Cross-section of left gonad of "Mule," no. 4, folded on itself. Shows large vacant spaces or cysts where ovarian follicles or oögonia have apparently been eliminated. ×84.

FIG. 25. Cross-section of left gonad of "Mule," no. 4, showing vacant "follicles" and infantile seminiferous tubules. ×84.

FIG. 26. Another section of left gonad to show other regions containing similar "follicles" and tubules. ×84.







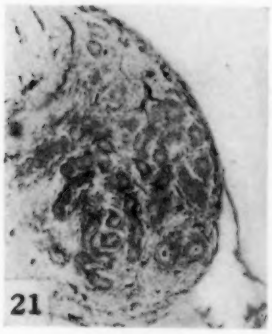


PLATE VI

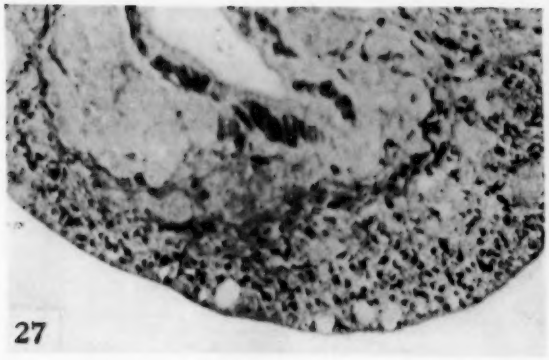
PLATE VII

FIG. 27. Cross-section near posterior end of gonad of "Mule," no. 4, showing one or two tubules and several vacant "follicles". ×84.

FIG. 28. Region of gonad of "Mule," no. 4, with two seminiferous tubules containing sertoli-like cells and cells that resemble spermatogonia. ×335.

FIGS. 29, 30, 31, 32, 33. Sections of the anterior end and region of the oviduct of "Mule," no. 4, in order antero-posteriorly. ×84.

FIGS. 34, 35, 36, 37. Succeeding cross-sections through the oviduct to show closure leaving two flaps and ventral groove and change in type of lining as sections are farther posteriorly. ×34.



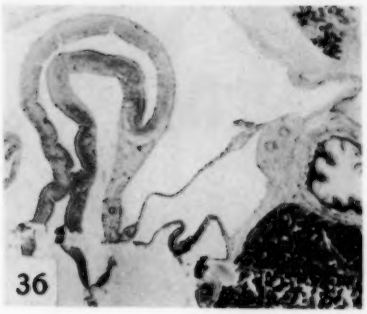
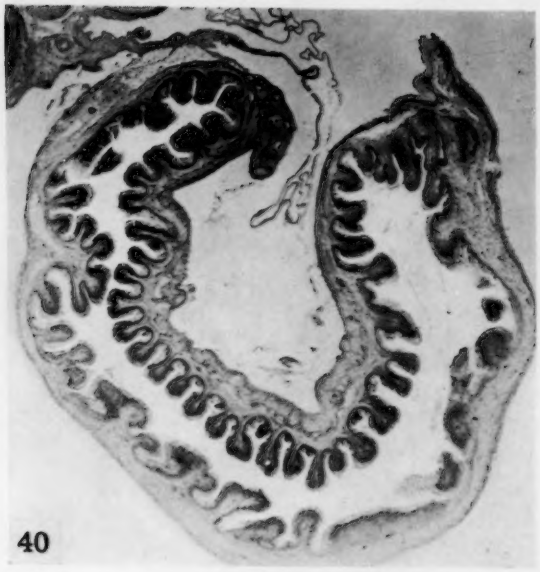


PLATE VII

PLATE VIII

FIGS. 38, 39, 40, 41. Succeeding sections of oviduct of "Mule," no. 4, leading almost to cloaca. Show change in type of lining and of the ventral flap or ridge. ×34.

38



40

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PLATE VIII

SOME PRE-ARMADA PROPAGANDIST POETRY IN ENGLAND (1585–1586)

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(Read April 26, 1941)

ABSTRACT

In the years 1585 and 1586, when England was expecting invasion by Spain, and combating "fifth-column" activities which centred around Mary Queen of Scots, the Oxford University Press issued a series of sixteen-page pamphlets intended to sustain the national morale. They were in Latin verse and appear to have had a wide distribution, but they have never been reprinted and are almost unknown to bibliographers. A set has been discovered at the Huntington Library and another at Winchester College, England. The most interesting of the contents are twelve odes, written during 1585 and 1586 and offering practically a month-by-month expression of national feeling.

THE year 1584 was one of great national apprehension in England. The Prince of Orange was assassinated on July 10 by a Jesuit agent, and similar plots against Queen Elizabeth that were being discovered in likely and unlikely places threw the country into a fury of excitement over what would now be called "fifth-column" activities. The Earl of Leicester, Chancellor of Oxford University, took the lead in forming the "bond of association" and in laying plans which culminated in the famous parliamentary act of November 23, "for the security of the Queen's person and continuance of the realm in peace".

In August, 1584, the University of Oxford directed that one hundred pounds be allowed to Joseph Barnes, a bookseller, in order that they might have a press in the University, and this was the real beginning of the Oxford University Press. Among the earliest, and today quite the rarest, of its publications is a series of sixteen-page pamphlets, issued in 1586 and 1587, and evidently intended to maintain the national morale. As befitted a learned press, they are in Latin, and in Latin verse of admirable quality. The first two, dated 1585, deal with the case of Dr. William Parry, who was executed as a traitor on March 2 of that year. One, entitled *Pareus*, treats the matter epically in hexameter. The other, *In Guil.Parry proditorem odae & epigrammata*, consists of separate poems. The three Horatian odes, from the pen of William Gager of Christ Church, the most accomplished Latin poet of this age, occupy six pages, and the rest are filled by a series of epigrams in elegiac verse against Parry, the Anglo-Romans, and the Pope.

The next year, 1586, brought more plots to kill the queen and more Horatian odes by Gager on the subject. Six of these, different from the three on Parry that had been printed the previous year, were published, again in a sixteen-page sheet, with the title, *In Catilinarias proditiones*. A wide sale was apparently expected, for the imprint notes that copies can be purchased in London, at the sign of the Tiger's Head in Paul's Churchyard.

The first is an impassioned malediction upon the evil days in which Gager is living. He would like to be borne beyond the Pillars of Hercules to the new world that Drake has visited, for the old world breeds traitors hydra-like, and the ancient British virtue has been polluted by Italian guile. He asks (if I may be permitted to rime his Latin):

> When will the headsman leave his work,
> And cleanse the stain of felon gore?
> When shall I see the axe at rest,
> And in the Tower no prisoners more?

The second ode is addressed to the most recently discovered of the Catholic plots, by which the navy and the city of London were to be set on fire. The poet has a vision of the queen sorely wounded and Britain scourged with flame and rapine. In the third ode he turns from the Alcaic stanza in which all the rest of this series are composed, to express in Sapphics his exultation at the queen's escape; and in the fourth he dilates upon the usual failure of treasonable plots and the miseries of a traitor's life. In Ode 5 he enforces with characteristic Old Testament instances the truth that regicide is the most impious of crimes; and in the sixth he urges the queen to have no mercy on traitors, pitiable as were the cases, he admits, of Tichborne and Salisbury, who were executed in September of this year. He incites Elizabeth to a supreme effort, like that of Jupiter against the rebellious Titans, and closes with impressive prophecy:

IN CATILINA-
RIAS PRODITIO-
NES, AC PRODITO-
RES DOMESTICOS, Ode 6.

OXONIÆ, ex Officina Typographica IOSEPHI BARNESII, & venenunt in Cœmeterio Paulino, sub signo capitis Tygurini. Anno 1586.

No poison, Queen, shall take you off,
No guile, revolt, or dagger stroke.
You'll gently pass, when ripe for fate:
Believe this, as if the Sibyl spoke.

Such work cannot but have been popular, and it is natural that a new issue of the odes was required before the year was out. By that time Gager had written three others, following the development of events, and these were squeezed within the limits of a sixteen-page pamphlet by printing the title on a separate leaf and beginning the text on signature A1 instead of A3.

The seventh ode is a sonorous vindication of the queen's character, and here for the first time Gager makes undoubted reference to Mary of Scotland, asking concerning his own queen,

When has *she* foully slain her mate?
Or when eloped? Not *hers* the guilt
Of wedlock fit for tragic stage,
And blood of folk and nobles spilt!

The next ode is addressed to the kings of Christendom, warning them in their own interests to destroy the English traitors who have found refuge in their realms; and the ninth brings the series to a fitting conclusion. It looks to the coming of the Armada, and appeals to Englishmen of every party to love their country and unite in resistance of all foreign domination.

The series of publications to which these odes of Gager belong has suffered the too usual fate of unbound, ephemeral literature. They have almost disappeared from the earth and have eluded the search even of the historians of the Oxford Press. Two sets of them, however, exist, bound up with other works. One passed through the hands of Richard Triplett, whose signature appears on the reproduced title-page, and who matriculated from Trinity College, Oxford in 1687, aged sixteen. It contains both the six-ode and the nine-ode edition of the poems printed below, and is now in the library of Winchester College, England. I am indebted to the Warden and Fellows of that School for permission to reproduce them and to the Librarian, Mr. C. E. R.

IN
CATILINARIAS PRO-
DITIONES, AC PRO-
DITORES DOMESTICOS,
Odæ 9.

OXONIÆ, ex Officina Typographica IOSEPHI BARNESII, Anno 1586.

Clarabut, for much courteous assistance. The other set, containing the nine-ode edition only, is in the Henry E. Huntington Library, to whose officers I must once more avow my gratitude. There are no important textual differences between the copies, though the later edition has been entirely reset.

ODAE 9,
ORNATISSI-
MIS VIRIS D. DO-
CTORI IAMESO, AE-
DIS CHRISTI OXON. DE-
CANO, ET D. DOCTORI HE-
TONO PRODECANO, CAETERIS-
QUE CLARISSIMIS ATQUE OP-
TIMIS VIRIS EIUSDEM EC-
CLESIAE PRAEBENDARI-
IS, ET PRIVATAE OB-
SERVANTIAE, ET
PUBLICAE PIE-
TATIS ERGO,
DICATAE.

In Domesticas, & immanes nostrorum hominum ac temporum proditiones,

Ode Prima

Nunc est per auras cum libet hinc rapi
Quacunque turbo praecipitem vehat,
Alcidis ultra vel columnas,
Magnanimosue Draci labores.

Nunc est sub antro quando libet tegi,
Ipsisque vitam degere cum feris,
Et glande vescentem caduca
Innocuis latitare Syluis.

Quo Proditoris nulla domestici
Pertingat unquam fama, ubi perfidus,
Laesaeque maiestatis unquam
Nemo mihi reus audiatur.

Quae dira, credam, toxica moribus
Aspersa nostris? tempora quae putem?
Quae terra? quae gens? quae libido
Non nisi grande nefas patrandi?

Faecunda culpae nostra quot edidit
AEtas Cethegos? quot modo Lentulos?
Iam creber urbi, Consulique
Insidias Catilina tendit.

Monstrumque maius, crimina pullulant
Dum puniuntur: iamque oritur scelus
Non unum in uno, sed resecta
Proditio, velut Hydra, crescit.

Ecquando ponet vincula carnifex?
Sontisque tabem sanguinis abluet?
Ecquando securas secures
Aspiciam, vacuamque Turrim?

O Christiani infamia seculi
Contracta nuper! proh scelus, et nefas
Non iam Britannis, sed vel ipsis
Turcigenis etiam pudendum.

Ode 1

Against the monstrous internal treasons of our citizens and times.

Now is a time when I wish to be snatched hence through the air, whithersoever the whirlwind can blow me headlong, past the Columns of Hercules or the daring voyages of Drake.

Now is a time when I wish to be shrouded in a cave and pass my life with the very beasts, to hide in the inoffensive woods and feed on the fallen acorn;

Where no news of the domestic traitor, can penetrate, where no disloyal person, and no man guilty of treason can ever be heard of by me.

What fatal poisons shall I believe instilled in our consciences? What times shall I think these to be? What an earth, what a race, what a passion for the accomplishing of nought but hideous crime!

How many Cethegi and Lentuli this sinful age of ours has lately produced! Not one Catiline, but many now spread their snares for the city and the chief magistrate.

And a greater horror: crimes propagate even while they are being punished; and now sin does not come singly in the single sinner, but treason grows like the hydra even as it is cropped.

O when will the executioner impose restraint, and wipe away the pollution of guilty blood? When shall I see the axes unemployed, and the Tower free of prisoners?

O newly devised infamy of this Christian age! Ah crime and sin, shameful now not merely to Britons, but even to the Turks themselves!

Nostri quis orbis, vel Tagus eluet
Nilusue tantam, vel Thamesis notam?
Non ipse qui cingit Britannos
Caeruleus pater expiarit.

Collecta, per tot secula, criminum
In nos redundat sarcina, perditeo
AEuo grauamur, quo malorum
Omne genus, scelerumque regnat.

Non his, iuuentus inclyta, moribus
Regno ferocem adiecit Hyberniam,
Gallumque et Hispanum fugauit
Non semel, indomitumque Scotum.

At nostra turpi dedita luxui,
Fortisque solam virginis in necem,
Artes dolosas tractat, ex quo
Italico medicata fuco est.

In horribilem, & plane Catilinariam
coniurationem nouissime factam,

ODE 2

Ergone si quid fraus poterat mala,
Extinctam Elisam lugubris Anglia
Iam fleret? arderentque naues?
Iam patriam cinis occuparet?

O impiorum viscera barbaro
Obducta ferro! posteritas fidem
Facti negabit, vix vel aetas
Nostra putet scelus institutum.

Regina tantum quid meruit pia?
Regina miti Candida pectore,
Diuina virgo, qua recurrens
Nil melius videt orbe Titan.

Non illa vobis, dira lues, placet:
Ast illa nobis perplacet, et Deo,
Sanaeque plebi, quam tuentur
Tot Procerum, populique vota.

Vos terra Diris prosequitur pijs,
Poscitque poenas immiserabiles;
Odere ciues, virginumque
Casta cohors scelus execratur.

Vix dum ipse tantos deposui metus;
Totus stupesco, membra quatit tremor,
Cum destinati saeua mentem
Forma mali subit, atque imago.

Detestor omen; sed video tamen
Crudeli Elisam vulnere sauciam,
Pulchros per artus ire cerno
Virginei lateris cruorem.

What Tagus, or Nile, or Thames will wash out this stain of our world? Not even the dark blue ocean-father who encircles us Britons could make us clean.

The weight of crime, heaped up through so many generations, pours upon us; we are weighed down by a desperate age in which every kind of evil and of sin is dominant.

It was with no such ideals as these that our splendid youth united fierce Ireland to our kingdom, and more than once put to flight the Gaul and Spaniard and the indomitable Scot.

But our youth of to-day, abandoned to base excess and bold only for the slaughter of a virgin, practices deceitful arts, from the time that it is besprinkled with Italian guile.

ODE 2

Against the horrible and quite Catilinarian conspiracy most recently perpetrated.

So, if wicked guile had its effect, distressed England would now be weeping for the slain Eliza, and our ships would be burning, and our fatherland reduced to ashes?

O bowels of sinners, covered with barbaric iron! posterity will refuse credence of your deed; hardly can even our age believe the crime you have purposed.

How has our noble queen deserved all this? Our queen so radiant with her tender heart, a heavenly virgin, than whom the circling sun sees nothing better in his course?

To you, ye deadly plague [of traitors] she is displeasing; but us she pleases much, and God, and her sound-hearted commons, and the manifold loyalties of her nobles and people protect her.

The earth pursues you with righteous portents and demands pitiless punishment; the citizens hate and the chaste band of virgins abhor your crime.

I myself have hardly yet thrown off these great alarms; I am all confounded, trembling shakes my limbs, when the horrid shape and idea of the plotted mischief comes into my mind.

I deprecate the omen; but yet I see Eliza smitten with a cruel wound, I behold the blood of her virginal breast flowing down her beautiful limbs.

Collapsa ceruix in niueos sinus
Laxe recumbit: sic ubi lilia
Succisa languescunt aratro,
Aut pluuia grauidum papauer.

Flagrare classem cerno Britannicam,
Urbes cremari, faemineos rapi
Artus capillo, virginesque
Et iuuenes trepidare inermes.

Quo tam impotenti Musa ruis pede?
Ah siste: res haec digna Philippicis,
Aut asperi diris Iambis
Archilochi, aut Senecae cothurno.

In Serenissimam Reginam Elizabetham ex imminente capitis periculo ereptam,

Ode 3

Quis mihi centum Deus ora, centum
Quis dabit linguas, totidemque voces
Ferreas, plenae pia quae refundant
Gaudia mentis?

Musa, dic cantus, age, seculares,
Carmen in Festo mihi seculari
Prome, quod longum, Dea, seculari
Viuat honore.

Quo rapis Cleio? videor repente
Iam super Pindo, gelidoue in Haemo,
Iamque Pernassi placido vagari
Vertice montis.

Ecce, quae cerno, loca grata, Tempe?
Quasque per ripas eo, quos per amnes?
Quis Caballini mea labra fontis
Proluit unda?

Proluor; nam quae mihi mens canendi,
Quis furor venit nouus, unde tantus
Ardor inuasit? libet incitatum
Fundere carmen.

Daulias non me Philomela cantu
Non olor vincet, licet illa tristes
Defleat casus, licet ille iam sit
Iam moriturus.

Versibus non me superet Catullus,
Nec lyra Flaccus, licet ille doctus,
Hic licet praestet Lyricis, et astra
Vertice tangat.

Quid moror? non me superabit Orpheus
Thracius, quamuis pater huic Apollo,
Nec Linus, quamuis Dea mater illi
Calliopea.

Her strengthless neck lies inertly upon her snowy bosom, just as where lilies languish when cut off by the plough or the poppy bent down with rain.

I behold the English fleet in flames, the cities burning, women dragged by the hair, and the helpless girls and boys in a panic.

Whither, Muse, are you rushing on such powerless feet? Ah, pause. This matter is worthy of Philippics, or the boding iambics of harsh Archilochus, or the buskin of Seneca.

Ode 3

Upon the most serene Queen Elizabeth, rescued from imminent peril of her life.

What god will give me a hundred mouths, a hundred tongues, and as many voices of iron, to pour out the loyal joys with which my mind is overflowing?

Come, Muse, recite songs for the ages; produce me on this historic festival a poem which may live long in age-old honour.

Whither are you taking me, Clio? Suddenly I now seem above Pindus, or on frosty Haemus, and now to be wandering on the quiet top of Mount Parnassus.

Lo, Tempe, what lovely landscapes I behold! and along what banks I go, beside what rivers! Who is it that washes my lips with water from the fount of Hippocrene?

I am washed clean; for what an urge to singing, what new mad impulse has come over me! Whence has so great a passion entered me? It is a joy to pour forth the rapid melody.

Neither the Phocian Philomela nor the swan shall surpass me in song, even though the one be bewailing her sad misfortunes and the other be on the point of death.

Catullus shall not excel me in versifying, nor Horace on the lyre, though the former is cunning, though the latter is preëminent among lyrists and touches the stars with his brow.

Why delay I? Thracian Orpheus shall not exceed me, though Apollo was his father, nor Linus, though his mother was the goddess Calliope.

Sospes exstructo (memorare dulce est
Quod pati durum nimis ah fuisset)
Sospes erepta est quasi fax Elisa
Nuper ab igne.

Quam malignorum prope funerarat
Turba periuris scelerata dextris,
Ni Deus tutam medios per enses
Eripuisset.

Ergo si remo mare defatigans
Seruus Hispano, medijsue Turcis
Degerem infaelix, Libycisue nudus
Syrtibus exul,

Annuos laeto tamen ore cantus
Funderem, ad remi modulantis ictum,
Muneris tanti memor, exararem
Carmen arena.

Iamque vix memet capio, libetque
Eloqui, mens est ululare syluis
Maenadum ritu, iuuat, et decorum est
Ire per antra.

Efferor; quid si minus appropinquet
Auribus diuae mea vox Elisae?
Audient colles tamen, et sonabunt
Undique rura.

Safe from the heaped up pyre—(how sweet to remember what [had it chanced otherwise] would have been too hard to endure!)—safe is Eliza, lately snatched like a brand from the burning.

Whom the impious band of malignants had nearly destroyed with their traitorous hands, had God not borne her safe from amid their swords.

Therefore, if I should live to be a galley-slave to the Spaniard, wearying the sea with my oar, or an unhappy captive among the Turks, or a naked exile on the Libyan shoals,

Yet with a happy voice I should pour forth my songs each year as the beat of my oar kept time, in memory of so rich a blessing, or should write out my poem in the sand.

And now I hardly can contain myself and have a joy in speaking out; my will is to shout in the forests after the fashion of the Bacchantes; it is pleasant and fitting to dance through the grottoes.

I am carried away. What though my voice can hardly reach the ears of the divine Eliza? Yet the hills shall hear, and the solitudes reecho on every side.

Voce campanas superabo septem,
Audiet longe, mediumque sese
Rumpet Hispanus, veluti solutus
Carmine serpens.

Audient dumi; neque vel leaenae
Vel lupi occursum metuam, vel apri,
Dum cano laetus nece liberatam
Elisabetham.

Me tegat mundi plaga inhospitalis,
Seu gelu squallens, nimioue sole,
Dulce erit saluae meminisse Elisae
Dulce referre.

Proditorum nefarios conatus
plerumque irritos esse,

ODE 4

Quo Proditorum stulta ruit cohors?
Quid spes inanes pascit, et impias?
Quid nectit occultos furores?
Et vacuas meditatur iras?

Ecquando vanos sentiet exitus,
Tristesque casus, illachrymabili
Fato trahentes se, domumque
Et proauos, sobolemque nequam?

My voice shall have more power than seven bells; far off the Spaniard shall hear it and burst amidships, like a serpent destroyed by a spell.

The thorn-bushes shall hear, and I shall fear to meet neither lioness nor wolf nor boar while happily I sing of Elizabeth saved from death.

Though some inhospitable zone of the world enclose me, hardened with ice or with excessive sun, it will be sweet to remember that Eliza is safe, sweet to repeat it.

ODE 4

That traitors' nefarious attempts
are commonly ineffectual.

Whither is the stupid congregation of traitors hastening? Why does it feed idle and impious hopes? Why does it weave secret furies and entertain empty rages?

When will it realize its vain results and doleful overthrows, which bring to unpitied ruin itself, its house and ancestors and worthless offspring?

Namque ut reuulsam seditionibus
Deleret urbem Gallus, et insolens
Hispanus, authores ad ipsos
Quanta mali quoque pars rediret?

Delectet hostem proditio, tamen
Quis Proditorem diligat, aut fide
Credat secunda, ex quo fefellit
Prima Deum, patriam, et penates?

Suspectus ergo turpiter exiget
Vitam, acriorem morte, sibi grauis,
Et seculi crimen, Deique
Ira furens, hominumque pestis.

Quid quod rebellem turris ahenea
Ut cingat, et nox vel pice nigrior
Occultet insanos tumultus,
Ante diem facinus patebit?

Claudat fenestras impius, et fores
Obducat intus mille, tegat leues
Rimas, recessu Dedalaeo
Implicitum Labyrinthus abdat:

Postes loquentur consilium tamen,
Eliminabit ianua, garrient
Ipsae volucres, et susurros
Ambiguos pia musca sparget.

For though the Gaul and haughty Spaniard should destroy our mutinous city, what a large part of the disaster would come home to the authors themselves!

Treason may please the enemy, but who can love the traitor or trust in a second fealty from one whose first has betrayed his God, his country, and his household?

Distrusted, therefore, let him in shame lead a life bitterer than death, a burden to himself and a blot on his age, the maddening wrath of God and plague of men.

Moreover, though a brazen tower surround the rebel, and a night blacker than even pitch conceal his mad revolts, before day his offence will lie revealed.

Let the offender close his windows, and lock himself within a thousand doors, let him cover the tiny cracks, and let a labyrinth conceal him, sequestered in a Daedalian maze:

Nevertheless, the door-posts will proclaim his secret, the gateway will blab it, the very birds will talk, and the dutiful fly will spread abroad dark whisperings.

Ne crede syluis, cum scelus apparas,
Aurita sylua est: ne domui tuae,
Muris ocelli insunt, domusque
Tota nefas videt, et recludet.

Ut nemo prodat: ne tibi, ne tuae
Confide menti: saepe tremens timor,
Horrorque vultus, saepe pallor,
Saepe stupor scelus occupauit.

Ut tute celes: quae tamen exedent
Curae, quis angor pectoris, et minae
Insomniorum? hic crede Manes,
Et triplices habitare Diras.

Omnem expauescit Proditor ad sonum,
Horret susurros, omnia dum putat
De se referri, seque nullo
Proripiet pede persequente.

Collum ille zona, pensilis a trabe,
Recte sequuta fregit, eum furor
Torsit, venenum absorpsit alter,
Hic proprio latus hausit ense.

Si cogitati tanta animum grauet,
Quae deprehensi est sarcina criminis?
At perpetrati quanta poena est?
Qui stimuli, furiaeque pungunt?

Put no faith in the forests when you plot a crime; the forest has quick ears: nor in your house; the walls have eyes, and the whole house witnesses your sin and will disclose it.

Though no one betray you, trust not yourself or your own mind: often tremulous fright and an agitated countenance, often paleness, often obsession, have betrayed sin.

Though you conceal it safely, yet what anxieties will consume you, what torment of soul and terrifying dreams? Believe me, here dwell the infernal ghosts and the threefold Furies.

At every sound the traitor trembles; he is aghast at whispers, thinking everything to have reference to himself, and he will flee away when no foot pursueth.

Dangling from a beam one has broken his neck with well employed girdle, madness has racked another, one has drunk poison, and another lanced his breast with his own sword.

If such a burden oppresses the mind for the meditated crime, what is the burden of the crime detected! And what the penalty for the crime committed! What goads and furies pierce the doer!

Ultor rebellem persequitur Deus,
Et cogitatos deserit exitus,
Ridetque fraudes, et nefando
Supplicium capiti reseruat.

In Proditores, summeque impium facinus esse
Principi violentas manus inferre,

ODE 5

Quisquamne credat? relligio nefas
Immane velat; tollere Principem
Coelo beatur, resque sancta est;
Grande scelus, pietas vocatur.

Hoc crimini unum defuerat, Deum
Facto aduocari: desine Proditor
Et numen insons inquinare,
Et Superos onerare probris.

Saulemne Dauid prodidit impium,
Iam certus haeres? quid simile est tamen?
Nec Saul Elisa est, nec, futuro
Vos geritis pia bella, Regi.

Is nescienti grande nefas putat
Scidisse vestem: vix cyathum manu
Hastamque tollit; vos Elisae
Et vigili, caput auferetis?

Is fata Saulis tristia flet pius,
Et nuntiantem morte remunerat,
Vel mentientem; vos Elisam
Fletis adhuc superesse terris?

Certe tyrannum, vel citus auferet
Morbus, vel ensis. quem patitur Deus
Nos ferre par est, et piorum
Arma, preces lachrymaeque sunto.

At Sheba Regem pessimus optimum
Conatur armis tollere subditis:
Dauidis ergo est non ferire,
Degeneris fera dextra Shebae est.

Ad Serenissimam Reginam Elizabetham, ne
Proditoribus parcat, eiusque salutem
Deo charam esse,

ODE 6

O Patre forti filia fortior,
Et matre pulchra filia pulchrior,
Regina, nostrarumque robur
Post Superos, columenque rerum:

Dic diua virgo, mens tibi quae fuit?
Vultus serenos quis subijt color,
Cum Proditorum prima nuper
Fama tuas violaret aures?

An avenging God pursues the rebel and turns his back upon his plotted ends, laughs at his wiles, and lays up punishment for his execrable head.

ODE 5

Against Traitors, and that the most impious of crimes is to lay violent hands upon a prince.

Can anyone believe it? Religion cloaks heinous crime! Regicide is blessed by heaven, and is a holy thing! enormous sin is called piety!

This alone was wanting to crime: that God should be made advocate of the deed! Cease, Traitor, to besmirch innocent divinity and load the higher powers with your sins.

Did David, now unquestioned heir, betray wicked Saul? What is there like in that? Neither is Saul Elizabeth, nor are you waging pious wars for the king to be.

David thinks it a great wrong to have cut off the skirt of Saul's robe privily, he hardly takes with his hand the cruse of water and the spear. Will you take off Elizabeth's head, even when she is on guard?

Godly David laments the sad fate of Saul, and rewards with death the announcer of it, even though a liar. Do you lament that Eliza is still present on the earth?

Certainly either quick disease or the sword will rid away the tyrant. [The ruler] whom God suffers, it is right for us to bear, and the arms of the righteous should be [merely] prayers and tears.

But a most wicked Sheba attempts to remove the best of kings by subject arms. Therefore, it is not for a David to strike; the hand of violence is the hand of degenerate Sheba.

ODE 6

To the most serene Queen Elizabeth, that she may not spare the traitors, and that her safety is precious to God.

O mightier daughter of a mighty father and fairer daughter of a mother fair! O Queen, next to the gods the strength and summit of our estate!

Say, divine virgin, what was your thought, what colour showed itself in your bright cheeks, when lately the first news of traitors shocked your ears?

Ecquid timebas? ecquid et in genis
Pallor sedebat? pectoris O fides:
Non tu timebas virgo multos,
Cerua canes, placida agna tygres.

Illud gemebas, quod fuerat tibi
Stringendus ensis; quid doleas tamen?
Effer secures, et nefandum
Poena comes scelus insequatur.

Tichburne (quaeso des veniam Anglia,
Et diua virgo) me miseret tui;
Dignaeque fato molliori
Salsberii miseret iuuentae.

Non illa mens est, non animus mihi
Mordere Manes; non ego mortuis
Insulto laetus, non cruoris
Ulla mihi subijt voluptas.

Odi cruentum supplicij genus;
Vester redundet sanguis ei in caput,
Qui crimen ingens suasit, at nos
Nulla grauet sobolemue culpa.

Sed charitates continet omnium
In se una cunctas patria nobilis.
Tichburne, iam nec me tui, nec
Salsberij miseret iuuentae.

Tu me seuerum patria, tu facis
Regina, durum: me capitis tui
Regina chari, me tuorum,
Me patriae miseret, meique.

Semperne strictas ut latus in tuum
Sicas timeres? tuta foris, pedem
Nunquamne figas? tune auito
Ut solio trepides inulta?

Nos, Proditoris ludibrium improbi
Et praeda, simus? nos miseros neci
Designet impune, et superbum
Instituat, duce te, triumphum?

Illi periclo dispereant suo
Nati gygantum sanguine; spiritus
Contunde sublimes, malumque
Eueniat male cogitanti.

Pindo Tiphoeus Pelion obruat,
Ossamque Olympi mole cacuminet,
Frendant Gygantes, et reuulsas
Enceladus iaculetur ornos:

Maior, trisulco fulmine, Iuppiter
Sternet rebelles, impiger aggeres
Euertet, aeternumque flammis
Sulphureae cruciabit AEtnae.

Did you fear aught? did any paleness show in your cheeks? O confidence of heart! You, a virgin, feared not the many men; a doe, feared not the dogs; a quiet lamb, the tigers.

At this you grieved: that you had to draw the sword. Yet why lament? Bring out the axes, and let meet punishment follow upon the accursed crime.

Tichborne, I pity you,—pray grant me pardon, England, and royal Virgin! And I pity the youth of Salisbury, worthy of a gentler fate.

That is not my spirit; it is not my disposition to gnaw at ghosts, I do not gladly triumph over the dead, nor have I ever felt any pleasure in blood.

I hate the gory type of punishment; let your blood flow upon the head of him who instigated this enormous crime, but let no guilt oppress us or your progeny.

Yet our one noble fatherland embraces in itself every devotion of us all. [In view of that,] Tichborne, I now pity neither you nor Salisbury's youth.

Fatherland, you make me harsh; Queen, you make me hard. What I pity, O Queen, is your dear head and your subjects, my country and myself.

Are you always to fear daggers unsheathed against your breast? Are you never to set foot abroad in safety? are you to tremble on your ancestral throne and have no redress?

Are we to be the jest and prey of a wicked traitor? Is he with impunity to mark us wretched ones out for slaughter and organize a proud triumph while you are Queen?

By their own peril let these sons of the giants' blood be undone; crush their arrogant spirits: Honi soit qui mal y pense!

Let Typhœus overwhelm Pelion with Pindus and set Ossa as a peak on the mass of Olympus; let the giants rage, and Enceladus hurl uprooted wild-ash trees:

Let Jupiter with his greater power lay low the rebels by three-forked lightning, let him strenuously overthrow their preparations, and torment them forever in the flames of sulphurous Ætna.

Titana proles consilij fere
Expers, ruinam mole trahit sua.
Odere Diui quicquid altum
Tentat iter, vetitumque nobis.

Caelo minatur qui solio inuidet:
Cognata regum est conditio Ioui:
Quos qui lacessit fraude, seu vi,
Ille Deum petit impotenter.

Unquamne surgat tam nigra lux mihi
Qua caesam Elisam vulnere defleam?
Non illa lux surget, Deusue
Tale nefas patietur unquam.

Nec te venenum, nec dolus auferet,
Nec sica, nec vis; molliter occides
Matura fatis, haec Sibyllam
Crede tibi cecinisse vatem.

Serenissimae Reginae nostrae Elizabethae, tot capitales perditorum hominum insidias, immeritissime indignissimeque factas esse,

ODE 7

Miretur aetas postera sanior,
Cur Proditores nostra tot extulit:
At cur Elisae tot pararunt
Exitium, merito stupescat.

Non Frater istas iam puer, aut Soror
Sensere fraudes, non Pater inclytus
Henricus armis, non, amicus
Pacis, Auus, proauique Reges.

Num Frater illam mente pia, Soror
Clemente vicit? num Pater ardua?
Prudentiae num laude neptim
Vicit Auus, proauique Reges?

Quando illa tantum commeruit nefas?
Qua parte peccat? quo scelere est nocens?
Tantas quid iras concitauit
Non nisi caede fera expiandas?

Quando illa dulcem saeua virum abstulit:
Quando reliquit? num tragico dedit
Dignas theatro nuptiales
Tot populi Procerumque strages?

Quem laesit unquam? quos nisi perdite
Ferro irruentes sponte sua, piger
Percussit ensis? queis libido est
Carnifices hebetare cultros.

Si quaerat illa; en, cuius ego bouem
Aut cuius agrum detinui, malum
Cui fraude feci? an non Britanni
Id referant? Nihil abstulisti.

This offspring of Titans, almost devoid of reason, invites ruin by its very size. The gods hate whatever attempts a too arrogant course and one forbidden to us.

He who is hostile to the throne is an assailant of heaven; the state of kings is allied to God; he who injures kings by fraud or force is ineffectually attacking God.

Can so black a day ever dawn for me whereon I am to mourn Eliza violently slain? No such day will dawn, nor will God ever suffer such an iniquity.

[O Elizabeth,] neither poison, nor trickery, nor dagger, nor force shall take you off. Gently you will die, ripened by the fates. Have faith that the prophetess Sibylla has made you this prediction.

ODE 7

That all these mortal plots of abandoned men against our most serene Queen Elizabeth have been made most undeservedly and unworthily.

A later, saner age will wonder why ours brought forth so many traitors. Yes, it may justly be astounded why so many attempted Eliza's death.

No such treasons did her brother experience, though but a boy; nor her sister; nor her war-famed father Henry; nor her grandfather, that friend of peace, and the preceding kings.

Did her brother surpass her in piety, or her sister in mercy, or her father in vigour? In fame of prudence was this scion inferior to her grandsire and the preceding kings?

When has she merited such outrage? In what point does she sin? of what crime is she guilty? What has engendered such hostilities, expiable only by savage slaughter?

When did she cruelly slay a tender husband? when did she abandon one? Has she been guilty of nuptials worthy of the tragic theatre and innumerable murders of populace and nobles?

Whom has she ever injured? Whom has her unwilling sword smitten, except those that of their own will rushed desperately upon her with their weapons, those who have a lust for blunting the knives of the executioner?

If she ask, 'Lo, whose ox have I taken, or whose field? whom have I defrauded?' may not the Britons answer thus, 'Thou hast not taken aught'?

Non illa Circe est, non animi impotens
Medea, ferro, perfide, quam petis?
Cur stringis ensem? siste, Elisa est,
Cui properas aperire pectus,

Quacunque curru sol habitabiles
Illustrat oras, optima principum:
Regina virgo, cui quid aetas
Nostra videt simile, aut videbit?

Percurre Reges, quos habet undique
Immensus orbis (liuor edax meis
Absit Camaenis) inter omnes
Luna micat, velut inter astra.

Cuius beauit tot meritis Deus
Tam mite regnum, dum misere fremunt
Armis, et ardent Marte gentes,
Alta suos tegit umbra pacis.

Priuata sceptro si posito foret,
Quis non Elisam diligeret tamen?
Cui non placeret? quis potenti
Imperio genitam negaret?

Quae forma vincens Sithoniam niuem?
Quae vita? quae mens? quae sine crimine
Dulcedo morum? absolueretur,
Inuidia licet assidente.

She is no Circe, no brainsick Medea. O traitor, whom are you threatening with your blade? why do you draw your sword? Stop. It is Eliza whose heart you strive to pierce,

The best of princes through all the lands that the sun lights with his chariot and makes habitable; a virgin queen, whose like our age sees not, and shall not see!

Consider the kings whom our boundless world possesses in all its parts. Far be carping belittlement from my verses, but she shines among them all like the moon amid the stars.

God has blessed her gentle reign with countless benefits. While the nations [abroad] groan miserably under arms and blaze with war, the deep shadow of peace enfolds her subjects.

If she should lay aside her sceptre and live as a private person, who would not nevertheless love Eliza? who would not admire her? who would deny that she was born for sovereign power?

What beauty, surpassing Thracian snow! How good a life, and what a mind! what blameless sweetness of disposition! She would be exonerated even though envy were her judge.

Laus ergo sane est magna, viri cadat
Si virgo dextra, si rapiat piam
Miluus columbam, vultur agnam,
Sanguineo lupus ore ceruam.

Gaudentne caesa virgine Caelites?
Haec placat illos victima? cur Deos
Praetendis insontes? Elisam
Cur gladio petis innocentem?

Crudele pectus, Proditor, exue:
Vagina ferrum condat, et excute
Sensus nefandos, et cruore
Parce pio scelerare dextram.

Ad Christianos Reges, ut transfugas
Proditores finibus suis exterminent,

ODE 8

Saluete Reges, progenies Deûm
Et certa proles, imperium quibus
Caelo secundum, vestra virtus
Et Superi faciles dederunt:

Seruate cauti sceptra, satellites
Fidi coronent; Proditio furit:
En sanguinis vestri nefanda
Quae subijt sitis hauriendi?

A noble exploit, then, it surely is, if the virgin fall by some man's hand, if the kite seize upon the gentle dove, the vulture on the lamb, the wolf with gory mouth upon the doe!

Do the immortals delight in a virgin's murder? does such a victim propitiate them? Why do you invoke the unsinning gods? why do you attack innocent Eliza with your sword?

Rid yourself, Traitor, of your cruel heart; let the sheath hold the dagger; renounce your execrable purposes, and desist from polluting your hand with righteous blood.

ODE 8

To the Christian kings, that they may exterminate the escaped traitors in their territories.

Hail, ye Kings, offspring and undoubted issue of the gods, to whom your own merit and the gracious immortals have given a power next to heaven:

Protect your sceptres carefully, and may faithful guards encompass you! Treason is raging. Behold, what an accursed thirst has grown to shed your blood!

Vos, Proditores hospitio procul
Arcete regni. perniciem trahent
Exempla vestris, totum ouile
Tabida ouis vitiabit una.

Quae grande mens est ausa nefas semel,
Claroque gaudens sanguine dextera
Nunquam quiescet, nec colorem
Lana bibit vitiata primum.

Impune per vos in dominans caput
Audebit oris Proditor improbi?
Impune maiestas iacebit
Quisquilijs hominum terenda?

Unita sors est tot dominantium;
Quenquam quod urit, quemque premat malum.
Ardente vicini camino,
Vestra domus caueat fauillas.

Quo Musa tendis? desine Regibus
Garrire. nostram summa breuis decet
Legationem, longiore
Purpureas onerabis aures.

Hear ye! Keep traitors far from the shelter of your kingdom. Their examples will carry the bane to your people; a single diseased sheep will corrupt the entire flock.

The mind that once has attempted a colossal crime, and the hand that craves the blood of rulers, will never rest; wool that has been stained will not take its original colour.

Shall the evil-faced traitor make his attempt upon a crowned head uncontrolled by you? shall majesty lie at the mercy of the dregs of mankind, with no redress?

The destiny of the many rulers is a joint destiny; the danger that afflicts any one may threaten every one. When your neighbour's chimney is afire, let your own house beware of sparks.

Whither are you ranging, Muse? Cease to prate to the kings. A brief compass fits our message; with a longer one you will weary their noble ears.

Ad nostrates Anglos, ut quibuscunque partium studijs ducti, externum dominatum omnes abominentur,

ODE 9

O Luce dulci Patria dulcior,
Et chara Tellus, pars animae meae
Non parua, ductricem Britannam
Compositis odijs sequamur.

Quaecunque nostras relligio imbuit
Mentes, Elisam praeferat extero.
Quaecunque discordes fatigat,
In patriam pia nulla suadet.

Quam solus ut tu mente regas tua
Vis esse nullam, Proditor? extera
Suspecta vis est, nec recedet
Turba valens, sitiensque regni.

Ut sana fiat Patria, sauciam
Hostine trades, qui solidas simul
Partes aduret, qui secabit?
Horribilis medicina bellum est.

ODE 9

To our English fellow-countrymen, that, with whatever zeal of party they are moved, they may all abhor the rule of foreigners.

O country sweeter than the sweet light of day, dear native land, a great part of my soul! Let us make up our quarrels and obey our English governess.

Whatever religion colours our minds, let it set Eliza above an alien. Whatever religion vexes us with discord, no righteous one impels us against our country.

Do you alone, Traitor, wish it annihilated, that you may rule according to your will? Foreign power is to be mistrusted, and a strong force, thirsting for sovereignty, will not withdraw.

To make our country well, will you deliver it wounded to the enemy, who will burn and slash its intact parts? War is a horrible medicine.

Quo prisca virtus, quo recidit vigor
Innatus Anglis, indocilis pati
Nutus heriles transmarini
Imperij, regimenque durum?

Hos miles agros, has segetes metet
Hispanus, auro, non numero valens?
Haec arua Gallo conseruntur?
Haec mea sunt, veteres coloni

Whither has departed from the English their former valour and the inborn robustness, incapable of suffering the lordly commands and hard yoke of an empire across the sea?

Shall the Spanish soldier, strong with gold, not men, reap these meadows and cornfields? Are these lands tilled for the Frenchman? 'These are mine,' shall the victor say?

Migrate, victor dicet? et insolens
Caedet morantes? nos taciti metu
Ceruice submissa, superbi
Turpe iugum domini feremus?

AEterna me nox ante, precor, tegat,
Ensisque tollat vulnere patrius;
Seruire fas O sit Britanno;
Seruitium domino leuatur.

'Move out, ye ancient occupants!' And shall he wantonly kill those who delay? Shall we, speechless with fear, bear with hanging heads the base yoke of an arrogant master?

Sooner, I pray, may eternal night enclose me, and my father's sword wound me to death! Be it my lot to serve a Briton! Servitude is alleviated by the [quality of the] master.

THE SHIP OF THE SOUL ON A GROUP OF GRAVE-STELAE FROM TERENUTHIS

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(Read April 26, 1941)

ABSTRACT

In this paper four small grave-stelae from Terenuthis in lower Egypt are illustrated, and their interpretation is discussed. The period of the burials was at the end of the fourth or the beginning of the fifth century of the Christian era. In all four examples a figure representing the deceased person is in a boat or is about to enter one. It is well known that the ancient Greeks often compared human life to a voyage, and the imagery connected with the ship of life was taken over by the Christians. In consequence of this, the ship-symbol occurs on a number of Christian tombs in the catacombs of Rome. In previously published material from Christian Egypt, this symbol is rarely found; and yet the Christian idea of the ship of the soul divinely guided to its final harbor was probably accepted all the more readily in Egypt, because the people had inherited from dynastic times the belief that the soul was carried in a boat to the abode of the blessed, sometimes with the guidance of a divine ferryman.

IN the spring of 1935 an expedition of the University of Michigan under the direction of E. E. Peterson excavated a cemetery of the late fourth and early fifth centuries of our era at Kom Abu Billu in lower Egypt. This place is thought to be the site of the ancient Terenuthis or the necropolis belonging to that town. It is just west of the western or Rosetta branch of the Nile, about ten miles from Kafr Daûd, and on the road to the Wadi Natrun. Among the objects discovered were a large number of limestone grave-stelae of moderate or quite small sizes; an area of 18 × 18 inches would accommodate almost any one of them, and many are much smaller. These stones were set into the grave-mounds themselves; Fig. 1 shows in its original position one of the stelae that will be examined in detail later. It is set in a sort of recess in one end of the built-up mound, as if it closed an entrance to the grave. The structure of some of these mounds is quite peculiar, but the description of them belongs to the report of the expedition and cannot be undertaken here.

The workmanship of these sculptures is poor. On several of the stelae, including the group to be discussed in this paper, the designs are outlined by the simple method of incision below the level of the original plane of the slab, and the background is not cut away. As a result, the shadows are deep, and detract not a little from the effect of the designs. Considerable traces of color remain on many of the specimens.

About 125 of these grave-stelae are now in the Museum of Classical Archaeology at the University of Michigan. Many of them show with somewhat monotonous regularity a design representing the deceased person reclining upon a couch at a banquet; he usually holds a cup in his extended right hand, and a table with various articles of food and certain other objects stand

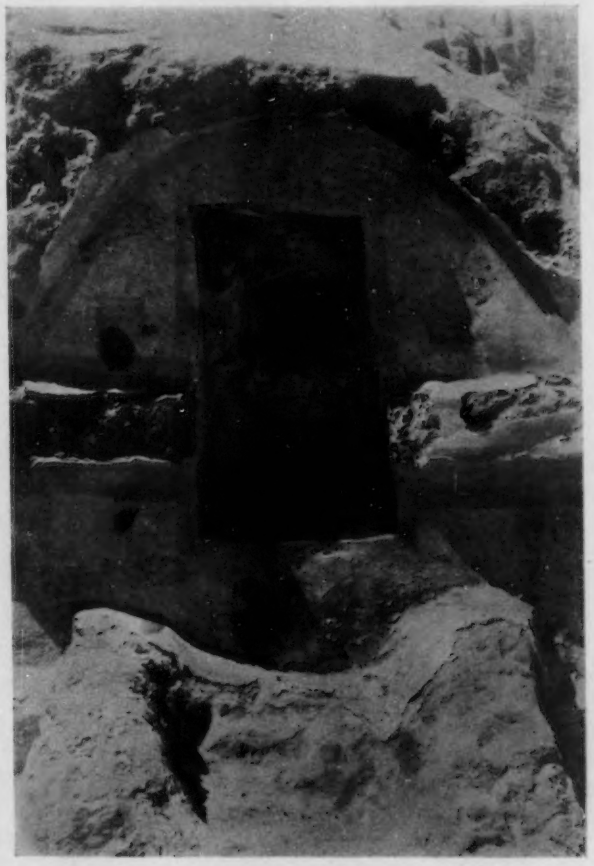

FIG. 1.

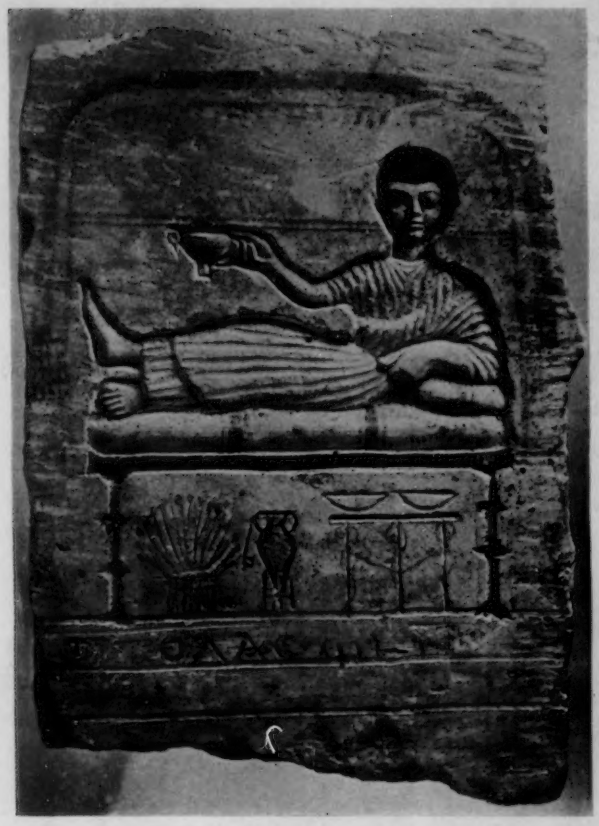

FIG. 2.

beside the couch[1] (Fig. 2: 29.7 cm. high, 20.7 cm. wide). We shall have occasion to return to this design later for a special purpose.

There are four stelae that present a different design, in which the figure of the deceased is either in a boat or is about to enter one. This group attracted my attention because I had recently been interested in the symbolism of the ship or boat; and I am publishing these four by the kind permission of the authorities of the Museum.

The inscription upon the first of these stelae (Fig. 3: 25 × 21 cm.) shows that it is the monument of a young man named Apion, twenty-six years old.[2] He is standing with both hands raised as if in prayer, an attitude known from many Christian monuments not only in Egypt but also in Rome.[3] He is in a small boat with steering-paddle, but no sail. Its shape is much like that of the boats so well known from the sculptures and paintings of dynastic Egypt. The work, which is very crude, continues a tradition of much earlier reliefs and paintings in representing the lower part of the body and the feet in profile although the figure faces front.

FIG. 3.

[1] Pictorial representations of the banquet in heaven are discussed by C. M. Kaufmann, *Die sepulcralen Jenseitsdenkmäler* (pp. 194–206, Die Darstellungen des himmlischen Gastmahles). The idea of feasting in the other world is of course very ancient, and doubtless grows out of a belief that the wants of the dead are like those of the living; Greek grave-reliefs of heroes feasting are well known. Leaders of the Orphic sect were said to promise their followers a banquet, and even eternal drunkenness, in the other world. The authorities are listed in Rohde, *Psyche*,[4] I, p. 315, n. 2, II, p. 129, n. 3; see also Guthrie, *Orpheus and Greek Religion*, pp. 158–160. For the "Hero-Feast" and its possible relation to the Orphic doctrine (with some cuts of typical reliefs), see Harrison, *Prolegomena to the Study of Greek Religion*,[2] pp. 349 ff., 614. There is little doubt that Greek reliefs depicting this subject exerted the principal formative influence upon the pattern of the Terenuthis stelae that represent the dead at a banquet.

[2] The second numeral, the so-called stigma, is so low and flat that the reading might be doubted; but the sign occurs in just such a form on many documentary papyri of the period.

[3] Examples from Roman monuments may be seen in Garrucci, *Storia dell' arte cristiana*, tav. 482, 485. Boldetti, *Osservazioni sopra i Cimiteri*, pp. 369, 372, 377, 378; for Egyptian examples, see Crum, *Coptic Monuments*, Pl. XLIX–LIII (Catal. gén. des antiquités égyptiennes du Musée du Caire, 1912).

The second is even cruder (Fig. 4: 28 × 22.5 cm.). Here the deceased is in a squatting position in the boat. There is no inscription; the angular cuts at the bottom are not letters but a suggestion of the ripples of the water. There are traces of red paint on the head, neck and arms of the man.

Next in order is a specimen that is something of an archaeological curiosity (Fig. 5: 14 × 20 cm.). Here the dead man is represented as reclining in a boat, which has a mast, yard and sail. His right arm is extended towards and a little beyond the mast, but is not, as might be thought at a careless glance, engaged with any

FIG. 4.

FIG. 5.

part of the tackle. As a matter of fact, the right hand holds a cup, as if he were at dinner; and thus it is apparent that the stone-cutter has simply combined two designs, and that very awkwardly. Taking the reclining figure from the often used pattern of the banquet, he has simply imposed it upon the boat; and in doing so he has placed the man with his feet to the paddle, in a position where he could not use it, even if one hand were not occupied with the incongruous cup, and he has neglected to bring the mast into connection with the hull of the boat.

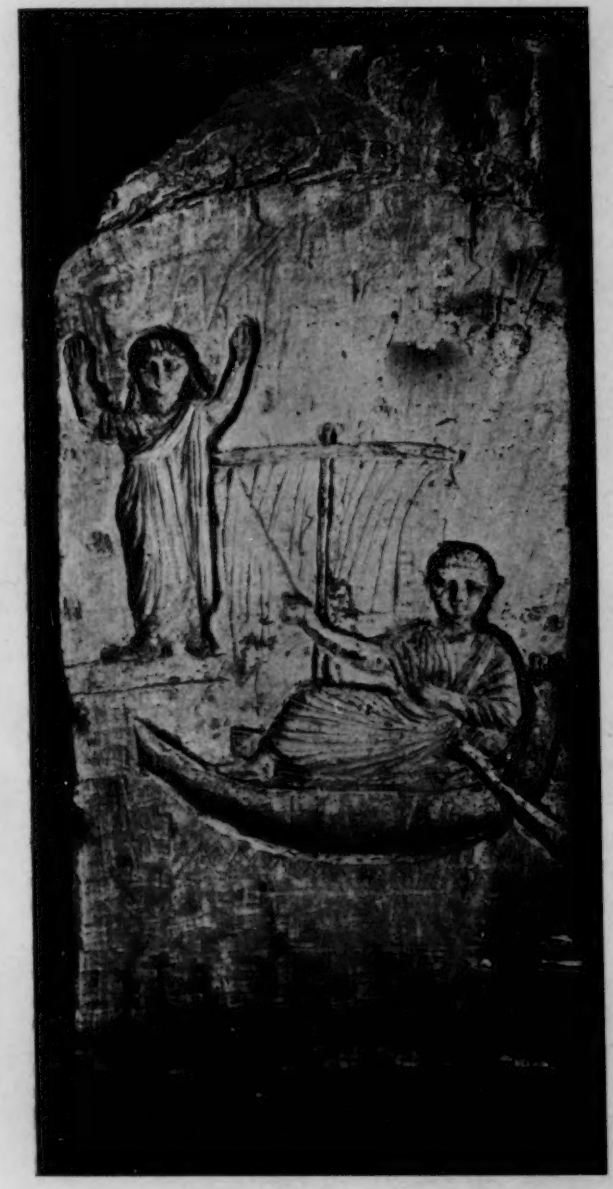

FIG. 6.

The fourth of these boat monuments has two parts in its design (Fig. 6: 50 × 23 cm.). In the left background, on the shore of a stream, stands a female figure with the hands raised in the posture of prayer. Her chiton, or undergarment, is a dark sea-green; all other colors are destroyed. Out in the stream is a boat similar in design to that seen in Fig. 3; in it sits a youthful male figure. Here also the design of the banqueter has had some influence; for the left elbow and part of the forearm of the man in the boat rest upon a cushion, as in the banquet-monuments, and the hand in consequence is held straight out instead of resting upon the steering-paddle. But the sculptor has given the right hand something to do. It seems to be drawing upon a rope attached to the yard as if to swing it around to a new position, probably, as we shall see later, in order to lay the boat alongside the shore. The water is indicated, as in a previous example, (Fig. 4) by an angular ripple.

Before dealing with the interpretation of this monument it is necessary to rehearse briefly what is known of the symbolism of the boat in Christian times, and that cannot be done without some reference to the occurrence of that symbolism in pagan literature. There is no need to go into details, for the topic has been treated, more or less fully, by several writers.[4] First, then, there is abundant literary evidence that from an early period the seafaring Greeks saw a certain analogy between the vicissitudes of human life and the dangers and hardships of a sea-voyage. Here we are concerned not so much with passages like Alcaeus' famous metaphor in which the strife-torn state is compared to a ship in a storm,

[4] As far as I know, nobody has attempted to gather all similes or metaphors in which a ship plays a part. J. Kahlmeyer, in his Greifswald dissertation, *Seesturm und Schiffsbruch als Bild im antiken Schrifttum* (Hildesheim, 1934), does not limit himself to figures involving storm and shipwreck, but touches also upon other images of nautical origin. In pp. 272–286 of F. J. Dölger's *Sol Salutis* a number of important literary and archæological treatments of the symbolic ship are cited; the author is particularly interested in the eastward direction of the ship in connection with the eastward position in Christian worship. W. Vollgraff, discussing the Delphic paean, has collected many examples of metaphors based upon the idea of a harbor (*Bull. de corr. hellén.*, XLVIII, pp. 178–186); and the present writer, in *Harvard Theol. Rev.*, XXXIV, pp. 49–67, has dealt more fully with the same subject and related ideas. This last paper will be referred to later by its title, "Desired Haven," and its pages.

as with utterances like Plato's phrase "this voyage of life," where he has in mind the individual rather than the community. The idea that a god might guide an actual ship and deliver the mariners from danger is found in Homer and Aeschylus, and philosophers talked of divine guidance in the voyage of life, although to some of them it seemed that it was merely the goddess of chance who steered the vessel of human fortunes. The last voyage of all ended in the harbor of death; and in a solemn passage of his *Seven Against Thebes* Aeschylus sings of the dark ship that passes over Acheron bearing its freight of souls to the sunless land. It will be remembered that according to a popular belief, the soul had a companion on this voyage across the dark water. This was the gloomy and morose Charon, a daemon of death, who ferried the soul across to its abiding-place.[5]

Thus far, then, we see that a boat guided by a daemonic ferryman played a part in a genuine popular belief of the Greeks, and that quite apart from that bit of folk-myth, both poets and philosophers often thought of life as a voyage, in which a divinity might be at the helm of the ship. In falling heir to pagan thought, Christianity naturally rejected the myth of the hateful Charon; but the figure of the voyage of life, or the ship of the soul, was not only unobjectionable in itself, but it could also be used for edification in connection with incidents of both Jewish and Christian sacred narratives. Some of the Church Fathers used the ark of Noah as a type of the Christian congregation, made up of men and women of all conditions; to others the story of Christ stilling the tempest on the Lake of Galilee suggested the image of the Church as a ship struggling with the storms of the world, with Christ as pilot, the apostles as officers, and all the members of the Christian body as crew or passengers. But along with the image of the ship as the church, there persisted the idea of the ship as the type of the individual life, guided by Christ as pilot and master, and that figure enters into a number of hymns and hortatory writings. It is not surprising that Clement of Alexandria mentions a ship sailing before a favorable wind among the devices that are appropriate for a Christian's seal-ring.[6]

This literary and theological tradition would seem to be enough to account for the use of the symbol of the ship in connection with Christian tombs anywhere. The archaeological material illustrating this symbolism, so far as it comes from Roman tombs and catacombs, has been collected and ably examined, especially by Catholic scholars.[7] A few examples of it may be cited as analogues to the Terenuthis stelæ. There is an inscribed slab from the cemetery of Prætextatus on the Appian Way, bearing the name of Aelia Eusebia, and below it a ship sailing to the right—that is, to the sunrise—with a figure in the bow, unfortunately indistinct.[8] Much better known is a famous bronze lamp in the form of a ship, now in the Uffizi in Florence.[9] In the bow stands a man in the attitude of prayer, while another sits holding the steering-paddle at the stern. To the mast is fastened a tablet with the inscription *Dominus legem dat Valerio Severo Eutropi vivas*. As the inscription suggests, and as the object is generally interpreted, the figure at the helm is Christ, and the man in the bow is Valerius Severus Eutropius, to whom the lamp may have been presented. Of other examples, perhaps the most interesting is a slab showing a ship approaching a harbor, which is indicated by the lighthouse just ahead of it.[10]

Since our stelae are from an Egyptian site, it is natural to inquire whether there were specifically Egyptian ideas or customs that rein-

[5] The passages to which this paragraph refers are as follows: Alcaeus, fr. 30 (Diehl); Plato, *Laws*, 803 b; Homer, *Odyss.*, 9. 142–143; Aesch., *Agam.*, 661–666; *Seven*, 854–860. A number of allusions to the haven of death will be found in "Desired Haven," pp. 51–53; and on pp. 57–59 the philosophical use of the figure is illustrated.

[6] A number of passages showing the symbolic use that Christian writers made of the ship, the harbor, and ideas connected with them are cited in "Desired Haven," pp. 59–64. For the ark as the type of the Church, Hippolytus, *Elench.*, 9. 12. 23 is important; for elaborate comparisons of the Church, with its institutions and its hierarchy, to the parts and the crew of a ship, see Hippolytus, *de Antichristo*, 59, and [Clem.], *Epist. ad Jacob.*, 14 (Patrologia Graeca, II, 49). Clement's words about the ship as a seal-device are to be found in his *Paedagogus*, 3. 59. 2.

[7] De Rossi, in Pitra, *Spicil. Solesm.*, III, p. 563; Wilpert, article "Schiff" in F. X. Kraus, *Realencyklop. der christl. Altertümer*; Kaufmann, *Jenseitsdenkmäler*, pp. 178–186.

[8] Published by Kanzler, *Nuovo bulletino di arch. crist.*, XV, p. 124, Fig. 8.

[9] Garrucci, *Storia dell' arte cristiana*, VI, tav. 469, 1; on a smaller scale, but more accurately reproduced in Kaufmann, *Handbuch der christl. Archäol.*,[2] p. 296, Abb. 147. Wilpert, *Die röm. Mosaiken*,[2] I, p. 303, thinks the helmsman must be Paul, rather than Christ, because of his baldness.

[10] Garrucci, *op. cit.*, tav. 486, 20.

forced the general Christian tendency to employ a ship as the symbol of a soul, and the answer is definitely affirmative. Many Egyptian towns had their cemeteries on the other side of the Nile, and the last journey of many a man was made in a boat. Further there is both monumental and textual evidence from various periods that the soul of the deceased was believed to be carried in a boat to the region of the blessed.[11] In particular certain chapters of the *Book of the Dead* mention the ferryman of Osiris, who conveys the soul that is "true of word" to the abode of the god. There are also special charms to secure for the soul the use of a boat for his voyage; so, for example, the ninety-ninth chapter of the Theban recension, "Of bringing a boat in the Other World," which has for its vignette in several papyri a picture of a boat with the dead person sitting or standing in it.[12] As for the divine companion, the soul is sometimes pictured in a boat with Ra and other deities. The divinity in a boat continued to be a familiar concept long after the dynastic age. In the Roman period we hear much of the great festival of the Ploiaphesia or *navigium Isidis*, in which the ship of Isis was launched; and many coins show the goddess standing upon her ship, holding a corner of the swelling sail.[13] As a final bit of evidence from Roman times I may mention the scores of amulet-stones with a representation of the infant Horus, or Harpocrates, seated upon a lotus in a papyrus boat.[14]

No country has ever been more tenacious of ancient beliefs and ancient ways than Egypt. The custom of mummifying the dead lasted for some time after the introduction of Christianity. Sacred animals of the old religion, such as the hawk, the cat, and the jackal, are sculptured upon some of the Terenuthis monuments; and at a period much later than that of the necropolis of Terenuthis the cross on Coptic grave-stones sometimes has the form of the ancient *ankh*, the hieroglyph of life. Hence there is much reason to think that the Greek-Christian idea of the voyage of the soul, with the ship as its symbol, was readily accepted by the Christians of Egypt, not as something entirely new, but as a new application of an ancient idea. Yet actual examples of the soul-ship seem to be rare in Christian Egypt, unless they are hidden among unpublished material, and that rarity may lend a special value to these rude grave-stones from Terenuthis.

We are now ready to consider the interpretation of the fourth of these boat-monuments (Fig. 6). Since there is no inscription, and since no other remains have been preserved to indicate whether the entombed body was that of a man or a woman, one might perhaps conjecture that the figure in the boat represents the soul of a dead man setting out on his voyage to the land of spirits, and that the woman on the shore is a mourner. But this seems to be contradicted by the fact that on very many of these monuments it is certainly the deceased who is represented with his hands raised in prayer. In the opinion of de Rossi and Kaufmann, the praying figures on Roman tombs regularly represent souls in a state of blessedness. The Egyptologist Ebers even conjectured that the frequent occurrence of these *orantes* on Coptic grave-monuments was partly to be explained by the influence of the old hieroglyph for the *Ka*, the double or personality-soul, which was indicated by a pair of arms raised in the position .[15]

It seems more likely, then, that the figure on the shore is that of the dead woman to whose tomb this stela belongs. Who, then, is the boatman? Certainly not the Charon of Greek popular belief, for he is regularly represented as a grim, uncouth elderly man. Nor is it likely to be John the Baptist, who, according to a curious Coptic sermon, received a strange commission from Christ: if any man had while living celebrated the commemoration of the Precursor, John should transport his soul in a golden boat across the river of fire in Amente.[16] Rather, we must turn for the explanation to the Church Fathers, who so often speak of Christ as the kindly pilot of the soul in life and death; thus we find the closest analogue to this monument in the

[11] Erman, *Die Religion der Aegypter* (1913), p. 217; Budge, *The Mummy*, pp. 463–466.

[12] Good illustrations in L. Speleers, *Le Papyrus de Nefer Renpet*, Pl. XX; E. Naville, *The Funeral Papyrus of Iouiya*, Pl. XXIII.

[13] Good illustrations may be seen in Alföldi's *A Festival of Isis in Rome* (Dissertationes Pannonicae, Ser. II, fasc. 7, 1937), Pl. I, 1, 2, 7; IV, 1, 2, 6; XVIII, *passim*.

[14] Since stones with this design are usually classed with the despised "gnostic" gems, the available illustrations are few as compared to the large number of existing specimens; see, however, *Catalogue of the Southesk Collection*, I, Pl. XV, n. 54; Budge, *The Mummy*, p. 330 and Pl. XXIV; *Journ. Egypt. Arch.*, XVI, pp. 6–9.

[15] Kaufmann, *Jenseitsdenkmäler*, p. 109; *Handb. der Christl. Arch.*,[3] p. 272, and n. 1; Budge, *The Mummy*, p. 340, n. 1.

[16] Budge, *Coptic Apocrypha in the Dialect of Upper Egypt*, p. 347.

bronze lamp in Florence that was mentioned a short time ago. The makers of these objects would have understood and appreciated the imagery of Tennyson's "Crossing the Bar".

Since the boat-symbol seems to be rare in Egypt of the later age, it may be worth while to mention the only other examples of it that have come to my attention. One, published by Dr. W. E. Crum in his *Coptic Monuments*, is the grave-stone of a monk named Peter (Fig. 7).[17]

FIG. 7.

Above are three crosses, right and left a *crux ansata*, in the middle a *crux monogrammatica* (derived from a combination of the letters X and P, for Christ). Below, a ship, not occupied by a human figure. Amidships a device that was probably meant for the monogram ☧, although the loop of the P is merely suggested by the line dividing the two panels, and one of the diagonals. It may have been originally shown by the use of paint. Besides this grave-stone, we find a ship used as one of the types of decoration for the pilgrims' bottles known as Menas flasks or ampullæ, because they were manufactured in great numbers to contain the holy water and holy oil dispensed at the shrine of St. Menas in the Mareotic desert.[18] But as used on these flasks the ship has no funerary suggestion; it is merely a symbol of the Christian life, like the seal-rings that Clement of Alexandria approved.

This discussion of the soul-ship may be closed with the pathetic words inscribed upon the tomb of a certain Cosmas.[19] The composer of the Coptic epitaph makes the dead youth say:

> O journey to a strange land, farther than ever before! O voyage in which it is hard to come to the other shore! The sea is wide, its waves are wild, and little is my boat—the youth of my body, the brief count of my years.

[17] No. 8574, Pl. XXX.

[18] See Kaufmann, *Die Menasstadt*, I, Pl. 96, 10, 11, 12.

POSTSCRIPT

After the foregoing paper had been sent to the editors some further material bearing upon the symbolism of the ship was brought to my attention, and I append it here. My colleague Professor Richard Ettinghausen referred me to Beyer and Lietzmann's *Die jüdische Katakombe der Villa Torlonia* (1930), Pl. 18*d*, an illustration of a ship that was scratched into the plaster of a *loculus*. The ship has a sail and two steering-paddles, but no person is seen on board, and there is no inscription. It would seem to be the symbol of the life-voyage or of the departed soul, but there is no definite proof of this. The reference to the soul-ship seems to me to be clearer in a monument to which Professor David M. Robinson called my attention. This is the funerary stela of Kallineikos, which was first mentioned by Professor Robinson (*Am. Jour. Philol.*, 27, 1906, 448), and was subsequently published by A. Salač (*Bull. de corr. hellén.*, 44, 1920, 354–357). At the top are two busts, one of a bearded man, the other of a boy. The main inscription has to do with the death of the older man, Kallineikos; but it also mentions his younger brother, who seems to have died some time before. At the bottom of the monument is a large ship followed by a quite small one; the rigging shows that this smaller vessel is a true ship, not a dinghy attached to the larger one. It seems fairly clear that the two ships of different sizes represent the souls of the two men of different ages, and do not, as Salač thought, refer merely to the fact that Kallineikos was a ship-master (*ναύκληρος*).

I am further indebted to Professor Ettinghausen for a reference to two peculiar mummy-paintings that were shown by the Brooklyn Museum in its exhibition held early in 1941, and

[19] Crum, *Coptic Monuments*, no. 8706, Pl. LV; Mallon, *Coptica* (Mélanges de la faculté orientale, Université Saint Joseph, V, 2), p. 125.* The date of the monument is 786 A.D.

subsequently published in the volume *Pagan and Christian Egypt*, Nos. 9 and 10; one of these belongs to the Boston Museum of Fine Arts, the other to the Brummer Gallery. The designs are almost identical—a portrait of the deceased above, holding a cup in one hand and in the other an object that is doubtfully described in the Boston painting as a sheaf of wheat, in the other as a garland. I incline to think that in both examples it is an ear, or bunch of ears, of wheat. Below is the "Sokaris bark flanked by jackals". Sokaris (Sokar, Seker) was a Memphite god of the dead who was more or less identified with, or even superseded by Ptah and Osiris (Erman, *Die Religion der Aegypter*, 26); the boat is well illustrated and discussed in Budge, *The Gods of the Egyptians*, I, 504–506. Thus these two paintings seem to carry on the tradition of the soul-ship which we find in the vignettes of the Book of the Dead.

Attention was called in the article mentioned above (*Harv. Theol. Rev.*, 34, 60–62) to the frequent occurrence of figures of speech based upon the ship or the haven in Manichaean psalms. A passage which might well have been cited there shows that the idea of Jesus in a boat was a fixed element in the Manichaean mythology; this is to be found in Epiphanius *Haer.*, 66, 31 (III, 71 in Holl's edition) in a list of the "emanations": αἱ δὲ προβολαὶ πᾶσαι, ὁ Ἰησοῦς ὁ ἐν τῷ μικρῷ πλοίῳ, καὶ ἡ μήτηρ τῆς ζωῆς, καὶ οἱ δώδεκα κυβερνῆται, καὶ ἡ παρθένος τοῦ φωτός, κτλ. See also a short paper by S. Eitrem, "De servatore mundi navis gubernatore" in *Collectanea Neotestamentica* (ed. A. Fridrichsen, Uppsala, 1940), IV, 5–8.

C. B.

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